Anti-inflammatory Effect of *Spirulina fusiformis* on Adjuvant-Induced Arthritis in Mice

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The present study was carried out to evaluate the anti-inflammatory effect of *Spirulina fusiformis* on adjuvant-induced arthritis in mice. Arthritis was induced by intra dermal injection of complete freund's adjuvant (0.1 ml) into the right hind paw of Swiss albino mice. *Spirulina fusiformis* (800 mg/kg/b.wt) was orally administered for 8 d (from 11th to 18th day) to arthritic animals after adjuvant injection. The anti-inflammatory activity of *Spirulina fusiformis* was assessed by measuring paw volume, body weight, levels of lysosomal enzymes, tissue marker enzymes and glycoproteins in control and experimental animals. In adjuvant-induced arthritic animals, the levels of lysosomal enzymes, tissue marker enzymes, glycoproteins and the paw volume were increased significantly. However the body weight was found to be reduced when compared to control animals. Oral administration of *Spirulina fusiformis* (800 mg/kg/b.wt) significantly altered these above physical and biochemical changes observed in arthritic animals to near normal conditions. Hence results of this study clearly indicate that *Spirulina fusiformis* has promising anti-inflammatory activity against adjuvant-induced arthritic animals.

Key words *Spirulina fusiformis*; adjuvant-induced arthritis; lysosomal acid hydrolase; glycoprotein

Rheumatoid arthritis is a chronic progressive autoimmune disorder characterised by symmetric erosive synovitis.[1] The exact etiology of rheumatoid arthritis remains unknown. It has assumed that either a foreign agent or some alteration in control of cellular responses is involved in the chronic persistent synovial inflammation.[2] This disease affects about 1% of the general population worldwide.[3] Conventional medicine, including treatment with steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and biological agents such as tumour necrosis factor alpha and interleukin-1 beta antagonists has only limited success against rheumatoid arthritis. Such therapies are helpful in controlling the symptoms of acute rheumatoid arthritis, but their effects on chronic, prolonged rheumatoid arthritis are unsatisfactory, associated with various side effects.[4] Research indicates that in the United States, 60—90% patients suffering from arthritis, particularly rheumatoid arthritis, used complementary and alternative medicine, predominantly herbal therapies.[5] Nowadays, increasing efforts are being directed towards traditional herbal medicines and plant derived foods for the development of drugs with long acting anti-inflammatory activity and minimum side effects.

Spirulina is blue green algae of the Oscillateriaceae family which grows naturally in warm climate countries and has been considered as supplement in human and animal food.[6] Man has made use of this alga for centuries because of its alimentary value; in the last decades *Spirulina* has been grown commercially in several countries including U.S.A., Thailand, Taiwan, Vietnam, China, India and Cuba.[7] It has been utilized as a source of protein and vitamin supplements, and has been sold as a health drink or pills in tablet form for more than 10 years without any undesirable effect on humans. Its safety for human consumption has also been established through numerous toxicological studies.[8] They have been found to be a rich source of vitamins, minerals, essential fatty acids and antioxidant pigments such as carotenoids.[9]

In addition, several studies show that *Spirulina* species exhibit various biological activities such as reducing body weight in obese human,[9] hepatoprotective,[10] antitumour,[11] antimicrobial,[12] strengthening immune system,[13] radio protective,[14] metalloprotective,[15] and anti-inflammatory effects.[16]

To the best of our knowledge, the anti-inflammatory effect of *Spirulina fusiformis* has never been reported in adjuvant-induced arthritis. Therefore, by considering the nutritive value of *Spirulina* as well as its various biomedical activities, we carried out this investigation to determine the effect of *Spirulina fusiformis* on adjuvant-induced arthritis in mice, a well-established model for rheumatoid arthritis. The chosen biochemical markers were glycoprotein components, lysosomal enzymes and marker enzymes of plasma, liver and spleen in control and in experimental animals. The changes in the paw volume and body weight were also determined.

MATERIALS AND METHODS

Animals Swiss albino mice, 25—30 g, of either sex were obtained from Tamilnadu Veterinary College, Chennai, India. They were acclimatized for a week in a light and tempterture-controlled room with a 12 h dark–light cycle and fed with commercial pelleted feed from Hindustan Lever Ltd. (Mumbai, India) and water was made available freely.

Drug The commercially available *Spirulina fusiformis* (a fine dark blue-green spray-dried powder) was obtained from, RECON Ltd, Bangalore, India and it was dissolved in 2% gum acacia solution to give aqueous suspension. This aqueous suspension of *Spirulina fusiformis* was used at a dose 800 mg/kg/b.wt, orally. All other reagents used were standard laboratory reagents of analytical grade and they are purchased locally.

Dosage Based on our preliminary studies with different dosages (200, 400, 600, 800 mg) of this drug, it was found that 800 mg/kg b.w dosage produced significant anti-inflammatory effect by reducing paw swelling in adjuvant-induced arthritic animals. Hence 800 mg/kg b.wt dosage was consid-

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ered for this study.

Experimental Design Mice were divided into four groups each comprising of six animals. Group I served as controls. In Group II, arthritis was induced by intradermal injection of complete freund's adjuvant (0.01 ml) into the right hind paw.[17] The adjuvant (Tuberculosis Research Center, Chennai, India) contained heat-killed *Mycobacterium tuberculosis* (10 mg) in paraffin oil (1 ml). Group III comprised of arthritic mice were treated orally with *Spirulina fusiformis* (800 mg/kg/b.wt), respectively for 8 d, from day 11 to 18 after the administration of complete freund's adjuvant. Group IV control mice were treated orally with *Spirulina fusiformis* alone, for 8 d.

On the 19th day, at the end of the experimental period, the animals were sacrificed by cervical decapitation and blood was collected. The liver and spleen were immediately dissected out and homogenized in ice-cold 0.01 M, Tris HCl buffer pH 7.4 to give a 10% homogenate. The tissue homogenate of spleen and liver were used for assaying the lysosomal enzymes, tissue marker enzymes and glycoprotein levels.

Arthritis was assessed by means of physical and biochemical measurements. Paw volume was assessed by measurement of the right ipsilateral hind paw by use of a vernier scale and body weight was recorded in both control and experimental animals.

Assay of Lysosomal Enzymes Acid phosphatase was assayed by the method of King[18] using disodium phenyl phosphate as the substrate. The enzyme activity was expressed as μmoles of phenol liberated/(min/mg/protein). β-Glucuronidase was assayed by the method of Kawai and Anno.[19] The substrate for the enzyme reaction was *p*-nitrophenyl β-*d*-Glucuronide and the enzyme activity was assessed in terms of μmoles of nitrophenol liberated/(h/mg/protein). *N*-Acetyl glucosaminidase activity was assessed by the method of Marhun[20] using 4-nitrophenyl-*N*-acetyl glucosaminide as the substrate and its activity was expressed as μmoles of *p*-nitrophenol formed/(h/mg/protein). The activity of β-galactosidase was assessed by the method of Rosenblit[21] using 4-nitrophenyl-*N*-acetyl galactopyranoside as the substrate and its activity was expressed as μmoles of *p*-nitrophenol liberate/(h/mg/protein). Protein content was measured by the Lowry *et al.*[22]

Estimation of Protein-Bound Carbohydrates For the glycoprotein analysis, a known amount of liver and spleen tissue were defatted by dissolving in hexane and taken in test tube, to which 1 ml of 2 N HCl was added, and the tubes were sealed. Hydrolysis was complete by keeping the sealed tubes in 100 °C for 16—18 h. After hydrolysis, the contents were neutralized with NaOH and made up to known volume, and aliquots were used for glycoproteins determination.

Hexose was estimated by the method of Niebes.[23] The neutralized sample was mixed with orcinol–H_2SO_4 reagent, heated at 80 °C, cooled and left in the dark for 25 min for colour development. The absorbance was read at 540 nm. Hexosamine was estimated by the method of Wagner.[24] The acetyl acetone reagent consisting of trisodium phosphate and potassium tetraborate with acetyl acetone, was added to the neutralized sample and boiled. After cooling, Ehrlich's reagent was added and the pink colour developed was measured at 540 nm. Hexuronic acid was estimated by the method of Bitter and Muir.[25] Fucose was estimated by the method of Dische and Shettles.[26] Sialic acid was determined by the method of Aminoff[27] with modifications by Niebes.[23] The neutralized sample was mixed with 0.25 M periodate (in 0.1 N H_2SO_4) and the reaction was inhibited after 30 min by arsenite solution. Then thiobarbituric acid was added and the contents were heated. The pink colour that developed on cooling was measured at 540 nm.

Estimation of Tissue Marker Enzymes Tissue enzymes namely aspartate transaminase and alanine transaminase were estimated by the method of King.[28] The enzyme activities were expressed as μmoles of pyruvate liberated/min/mg/protein. Alkaline phosphatase was estimated by the method of King.[18] The activity of alkaline phosphatase was determined by measuring the phenol liberated from disodium phenyl phosphate by the colour reaction with folin's reagent in an alkaline pH. The activity of alkaline phosphatase was expressed as μmoles of phenol liberated/min/mg/protein.

Statistical Analysis Results were expressed as mean$\pm$S.D. and statistical analysis was performed using ANOVA, to determine significant differences between groups, followed by student's Newman–Keul's test. $p<0.05$ implied significance.

RESULTS

Figure 1 shows the effect of *Spirulina fusiformis* on paw volume in control and experimental animals. Measurement of the paw volume of mice with adjuvant-induced arthritis revealed an increase in ankle diameter from day 4, which increases further up to 19th day. *Spirulina fusiformis* treatment reduces the paw diameter significantly in adjuvant-induced arthritic mice.

Figure 2 shows the body weight changes of the control and experimental mice. The growth of arthritic mice (Group II) was found to be retarded. The body weight of *Spirulina fusiformis* treated arthritic animals (Group III) were found to increase when compared to that of control animals.

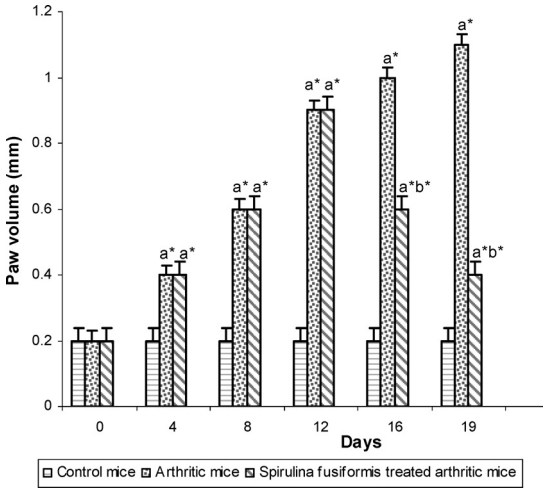

Fig. 1. Effects of *Spirulina fusiformis* on Paw Volume in Adjuvant-Induced Arthritic Mice

Values are expressed as mean$\pm$S.D. of six animals. Comparisons were made as follows: a, control mice *vs.* arthritic mice and *Spirulina fusiformis* treated arthritic mice; b, arthritic mice *vs. Spirulina fusiformis* treated arthritic mice. The symbols represent statistical significance at: $*p<0.05$.

Table 1 depicts the activities of lysosomal enzymes in the plasma, liver and spleen of control and experimental animals. In adjuvant-induced arthritic animals, the activities of acid phosphatase, β-glucuronidase, *N*-acetyl glucosaminidase, and β-galactosidase were increased significantly when compared to control animals. The administration of *Spirulina fusiformis* to arthritic mice significantly reversed the above changes to normal level considerably.

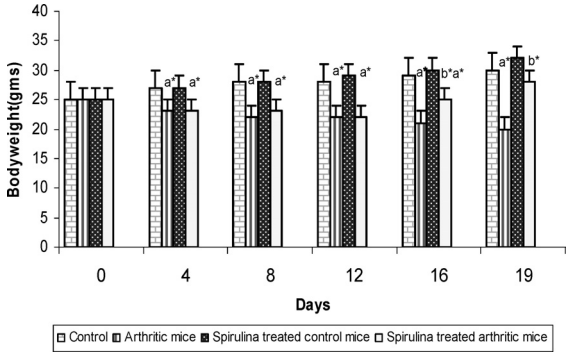

Fig. 2. Effect of *Spirulina fusiformis* on Body Weight Changes

Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: a, control mice *vs.* Arthritic mice, *Spirulina fusiformis* treated control mice and *Spirulina fusiformis* treated arthritic mice; b, arthritic mice *vs. Spirulina fusiformis* treated arthritic mice. The symbols represent statistical significance at: $*p<0.05$.

Table 2 depicts the effect of *Spirulina fusiformis* on the glycoprotein levels in liver and spleen of control and experimental animals. The sugar components of glycoproteins-hexose, hexosamine, hexuronic acid, sialic acid, and fucose were significantly increased in plasma and spleen of arthritic animals than control animals. *Spirulina fusiformis* administration to arthritic mice decreased the level of glycoproteins significantly.

Table 3 shows the effect of *Spirulina fusiformis* on the levels of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase in the control and experimental animals. A marked increase in amino transferases and alkaline phosphatase were observed in Group II arthritic mice in serum, liver, and spleen. *Spirulina fusiformis* treatment to arthritic mice (Group III) showed significant decrease in the

Table 1. Effect of *Spirulina fusiformis* on the Activities of Lysosomal Enzymes

Parameters	Group I Control	Group II Arthritis	Group III Arthritis+*Spirulina fusiformis*	Group IV *Spirulina fusiformis* control
Plasma				
Acid phosphatase	0.11±0.01	0.81±0.01 a*	0.12±0.01 b*	0.1±0.01
β-Glucuronidase	1.56±0.07	5.30±0.6 a*	1.98±0.10 b*a*	1.59±0.08
N-Acetyl glucosaminidase	1.93±0.11	3.11±0.13 a*	2.0±3 0.11 b*a*	1.96±0.11
β-Galactosidase	0.9±0.09	2.95±0.08 a*	1.20±0.08 b*a*	0.97±0.11
Liver				
Acid phosphatase	2.20±0.01	3.40±0.08 a*	2.04±0.11 b*	2.12±0.07
β-Glucuronidase	25±0.81	40.6±1.37 a*	30±0.87 b*a*	25.8±0.89
N-Acetyl glucosaminidase	28 ±0.81	39.5±0.95 a*	30.1±1.06 b*a*	28.8±0.68
β-Galactosidase	10±0.81	19.1±0.68 a*	12.83±0.68 b*a*	10.8±0.89
Spleen				
Acid phosphatase	2.86±0.07	4.5±0.08 a*	3.15±0.17 b*a*	2.9±0.05
β-Glucuronidase	29±0.81	46.5±0.95 a*	31.5±0.95 b*a*	29.16±1.06
N-Acetyl glucosaminidase	19.5±0.95	30.5±0.95 a*	22.33±1.24 b*a*	19.8±0.68
β-Galactosidase	5.1±0.6	10.25±0.69 a*	6.2±0.34 b*a*	5.33±0.52

Values are expressed as mean±S.D. of six animals. Treatment of groups are as follows: Group I, control; Group II, arthritic; Group III, arthritic mice treated with *Spirulina fusiformis* (800 mg/kg/b.wt) for 8 d; Group IV, control mice treated with *Spirulina fusiformis* for 8 d. Comparisons were made as follows: a, Group 1 *vs.* Group II, III, IV; b, Group II *vs.* Group III. Enzyme activities are expressed as: acid phosphatase: μmoles$\times 10^{-2}$ of phenol; β-glucuronidase, *N*-acetyl glucosaminidase, and β-galactosidase: μmoles$\times 10^{-2}$ of *p*-nitrophenol liberated /h/mg protein. The symbols represent statistical significance at: $*p<0.05$.

Table 2. Effect of *Spirulina fusiformis* on Protein Bound Carbohydrates

Parameters (mg/100 mg defatted tissue)	Group I Control	Group II Arthritis	Group III Arthritis+*Spirulina fusiformis*	Group IV *Spirulina fusiformis* control
Liver				
Hexose	2.10±0.12	3.90±0.25 a*	2.50±0.20 b*a*	2.20±0.15
Hexosamine	0.15±0.01	0.33±0.20 a*	0.20±0.01 b*	0.16±0.02
Hexuronic acid	0.30±0.02	0.57±0.04 a*	0.40±0.03 b*	0.32±0.02
Fucose	0.64±0.05	1.51±0.12 a*	0.90±0.07 b*	0.60±0.04
Sialic acid	25.50±2.00	40.10±3.82 a*	30.00±2.10 b*	27.00±1.8
Spleen				
Hexose	1.80±0.10	3.52±0.24 a*	2.10±0.15 b*	1.85±0.12
Hexosamine	0.20±0.01	0.30±0.02 a*	0.25±0.01 b*	0.19±0.01
Hexuronic acid	0.22±0.01	0.38±0.04 a*	0.21±0.01 b*	0.26±0.15
Fucose	0.51±0.04	0.86±0.07 a*	0.65±0.05 b*	0.55±0.04
Sialic acid	20.00±1.64	33.80±2.30 a*	25.00±2.00 b*	21.00±1.50

Values are expressed as mean±S.D. of six animals. Treatment of groups are as follows: Group I, control; Group II, arthritic; Group III, arthritic mice treated with *Spirulina fusiformis* (800 mg/kg/b.wt) for 8 d; Group IV, control mice treated with *Spirulina fusiformis* for 8 d. Comparisons were made as follows: a, Group I *vs.* Group II, III, and IV; b, Group II *vs.* Group III, The symbols represent statistical significance at: $*p<0.05$.

Table 3. Effect of *Spirulina fusiformis* on the Activities of Marker Enzymes

Parameters	Group I Control	Group II Arthritis	Group III Arthritis+*Spirulina fusiformis*	Group IV *Spirulina fusiformis* control
Plasma				
ALT	0.50±0.06	1.00±0.09 a*	0.65±0.06 a*b*	0.52±0.06
AST	0.42±0.05	0.09±0.08 a*	0.51±0.04 a*b*	0.45±0.05
ALP	2.10±0.20	5.10±0.45 a*	2.65±0.30 a*b*	2.15±0.25 a*
Liver				
ALT	0.09±0.05	0.26±0.04 a*	0.16±0.04 a*b*	0.11±0.09 a*
AST	0.10±0.04	0.23±0.03 a*	0.17±0.05 a*b*	0.12±0.08 a*
ALP	1.09±0.09	3.12±0.13 a*	1.56±0.18 a*b*	1.12±0.10 a*
Spleen				
ALT	0.11±0.01	0.30±0.02 a*	0.25±0.05 a*b*	0.15±0.01 a*
AST	0.15±0.06	0.45±0.03 a*	0.35±0.04 a*b*	0.19±0.09 a*
ALP	0.45±0.03	0.80±0.09 a*	0.58±0.05 a*b*	0.50±0.04 a*

Values are expressed as mean±S.D. of six animals. Treatment of groups are as follows: Group I, control; Group II, arthritic; Group III, arthritic mice treated with *Spirulina fusiformis* (800 mg/kg/b.wt) for 8 d; Group IV, control mice treated with *Spirulina fusiformis* for 8 d. Comparisons were made as follows: a, Group I *vs.* Group II, III, and IV; b, Group II *vs.* Group III. Enzymes units: Amino transferase: μmol$\times10^{-2}$ of pyruvate; ALP: μmol$\times10^{-2}$ of phenol. The symbols represent statistical significance at: $*\ p<0.05$.

level of marker enzymes, when compared to arthritic control mice (Group II).

DISCUSSION

In the present study, we investigated the anti-arthritic effect of *Spirulina fusiformis*, blue green algae, in adjuvant-induced arthritic mice. Results showed that *Spirulina fusiformis* significantly inhibited the paw swelling and this inhibition was accompained by a decrease in the levels of lysosomal acid hydrolases, tissue marker enzymes and glycoproteins. It has been well established that inflammation and lysosomal enzyme activities were increased after adjuvant injection.[29)]

Increased paw swelling observed in the arthritic mice was found to be a result of oedema of periarticular tissues. An increase in granulocytes and monocytes has been found to be associated with changes in ankle diameter.[30)] Paw swelling was significantly reduced in arthritic mice treated with *Spirulina fusiformis*, which indicates its interference on cyclo-oxgenase pathway.

Changes in the body weight are a useful index to assess the course of the disease and the response to therapy of anti-inflammatory drugs in quest.[31)] The loss of body weight observed in arthritic animals may be due to the reduced absorption of glucose and leucine in rat intestine.[32)] The increase in body weight during *Spirulina fusiformis* administration reveals the restoration of absorption capacity of the intestine in the arthritic animals. This increase in absorption capacity could be due to the presence of vitamins, minerals and antioxidant pigments in our drug.[9)]

A characteristic feature of adjuvant-induced arthritis in rats is the correlation between the development of inflammation and the release of lysosomal enzymes into the extra cellular compartment.[33)] In adjuvant-induced arthritis, the glycohydrolases released from invading macrophages, neutrophils, and from tissue cells such as synoviocytes and chondrocytes, initiates the synthesis of inflammatory mediators (thromboxanes, prostaglandins, and leukotrienes) which plays an important role in the rheumatoid process.[34)] Reduction of the release of lysosomal enzymes would prove beneficial and this indirectly confirms the protective effect of the drug. *Spirulina fusiformis* administration decreases the lysosomal enzyme release in adjuvant-induced arthritic mice, which indicates its anti-inflammatory effect.

Glycoproteins are the components of connective tissue that are responsible for the antigenic property in tissue transplants and maintaining the structural stability of collagen fibrils.[35)] The altered glycoproteins metabolism observed in arthritic animals (Group II) is due to the increased release of acid hydrolases during arthritic condition. These enzymes are involved in the degradation of structural macromolecules in connective tissue and cartilage proteoglycans. The metabolic turnover was found to be increased in the ligament and cartilage during the inflammatory process of adjuvant-induced arthritis.[36)] After *Spirulina fusiformis* treatment, glycoprotein levels were significantly decreased which may be due to its modulating role on lysosomal hydrolases.

Tissue damage was assessed by measuring the activities of enzyme in the serum and in the respective organ, since liver impairment is also a feature of adjuvant arthritis.[37)] The increase in aminotransferases is due to their release from the cells of the damaged organ.[30)] Subrata (1994) reported a similar increase in aminotransferases was observed in arthritic animals.[38)] Alkaline phosphatase has been reported to be present mainly in the blood vessels, pia arachnoid and choroid plexus. Alkaline phosphatase activity has been reported to increase during the morphological and functional development of the tissues. Aminotransferases and alkaline phosphatase were significantly reduced in arthritic mice after the administration of *Spirulina fusiformis*. This reducing effect may be related to their anti-inflammatory activity.

So far the results of the present study conclude that *Spirulina fusiformis* is able to suppress the changes produced during adjuvant-induced arthritis. This suppressing effect of *Spirulina fusiformis* could be due to its antioxidant constituents such as β-carotene,[39)] Vitamins C, E,[40)] and other micronutrients.[41)] However, further investigation on its effectiveness and mechanism are warranted before *Spirulina fusiformis* to be used as a supplement for the treatment of rheumatoid arthritis and is on the way.

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