Final Progress Report

Project entitled "Evaluation of anti-arthritic properties of *Spirulina platensis* in Collagen Induced Arthritis (CIA) in rats"

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Abbreviations

%	:	Percent
^0C	:	Degree centigrade
AAP	:	Aminoantipyrine
ALP	:	Alkaline phosphatase
ANOVA	:	Analysis of variance
Ca SP	:	Calcium Spirulan
CAD	:	Coronary artery disease
CCl_4	:	Carbon tetrachloride
CFA	:	Complete Freunds Adjuvant
CIA	:	Collagen induced arthritis
ConA	:	Concavalin A
COX-2	:	Cyclooxygenase-2
CTL	:	Cytotoxic T- lymphocytes
DA	:	Dark agaouti
DMARDs	:	Disease modifying antirheumatic drugs
DMSO	:	Dimethyl sulphoxide
DTH	:	Delayed type hypersensitivity
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
Fig.	:	Figure
g	:	Gram
HLA	:	Human leukocyte antigen
HRPO	:	Horse radish peroxidase
i.p.	:	Intraperitoneal
IFA	:	Incomplete Freunds Adjuvants
IL	:	Interleukin
Kg	:	Kilogram
KLH	:	Keyhole limpet hemocyanin

l	:	Liter
LPO	:	Lipid peroxidation
LTB_4	:	Leukotriene
mAb	:	Monoclonal antibody
MDA	:	Malondialdehyde
mg	:	Milligram
MHC	:	Major Histocampatibility complex
ml	:	Mili liter
mm	:	Milimeter
MPO	:	Myloperoxidase
MRI	:	Magnetic resonance imaging
MTX	:	Methotrexate
NK	:	Natural killer
NO	:	Nitric oxide
NSAIDs	:	Nonsteroid anti-inflammatory drugs
OA	:	Osteoarthritis
OD	:	Optical density
OPD	:	Orthophenylene diamine hydrochloride
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffer saline
PBST	:	Phosphate buffer saline-Tween 20
pg	:	Picogram
PGE_2	:	Prostaglandin
PIP	:	Proximal interphalangeal
PMN	:	Predominantly neutrophil
PUFA	:	Polyunsaturated fatty acids
RA	:	Rheumatoid arthritis
RF	:	Rheumatoid factor
ROS	:	Reactive oxygen species
RPMC	:	Rat peritoneal mast cells

RPMC	:	Rat peritoneal mast cells
SOD	:	Superoxide dismutase
TBA	:	Thiobarbituric acid
TEP	:	Tetra-ethoxypropane
TNF	:	Tumor necrosis factor
YLD	:	Years lived with disability
α	:	Alpha
β	:	Beta
µl	:	Micro liter

Introduction

Rheumatoid arthritis (RA) is characterized by a chronic inflammation of synovium, leading to progressive joint destruction. Erosions of the periarticular bone, the most specific hallmark of the disease, produce deformation, laxity, and functional disability. Local and systemic inflammation also favors generalized osteopenia or osteoporosis. Osteoclasts are considered as the principal cell type responsible for focal bone resorption in RA (Benoit *et al.* 2009). Thus, RA was a form of joint inflammation that resembled rheumatic fever. RA by definition is polyarticular; that is, it affects many joints. Most commonly, small joints (including the hands, feet and cervical spine) are affected, but larger joints (shoulders, knees etc.) can also be involved; the pattern of joint involvement can differ from patient to patient (Majithia and Geraci, 2007).

Kinne *et al.* (2007) stated that females are affected by RA at a ratio of approximately 3:1 compared with males and experience clinical fluctuations during the menstrual cycle and pregnancy, indicating a major modulating role for sex hormones. Due to expression of their sex-hormone receptors and their cytokine response upon exposure to estrogens, monocytes/macrophages are strongly involved in hormone modulation of RA (Cutolo and Lahita, 2005). Indeed, physiological levels of estrogens stimulate RA macrophages to the production of the pro-inflammatory cytokine IL-1, whereas, higher levels inhibit IL-1 production, conceivably mimicking the clinical improvement during pregnancy. Interestingly, selective estrogen receptor ligands inhibiting nuclear factor (NF)-κB transcriptional activity (but lacking estrogenic activity) can markedly inhibit joint swelling and destruction in experimental arthritis (Keith *et al.* 2005).

RA is the commonest inflammatory arthropathy worldwide that affects up to 0.75% of the Indian population (Malaviya *et al.* 1993). The disease follows a chronic course and the outcome may be unsatisfactory despite treatment. This is because the etiology of RA is not known and the pathogenetic mechanisms remain yet to be fully elucidated. Indian scientists have shown a greater association of HLA DR4 with RA. The HLA DR4-DQw8 haplotype occurs more commonly in RA rather than the HLA DR4-DQw7 haplotype seen in the West. In southern India, HLA DQw2 is commonly associated with RA, while HLA DQw1 occurs more often in northern Indian RA patients (Taneja *et al.* 1992). Seropositivity

for RF and/or severity of RA does not correlate well with the presence of HLA DR4 in northern and southern Indian patients (Porkodi and Chandrasekaran, 1989) and this is in contrast to the reports from the West (Taneja *et al.* 1992). In RA, normally the acellular joint is heavily infiltrated with a variety of leukocytes. A pannus is infiltrated with endothelial cells, synovial cells (fibroblast-like and macrophage like), B cells and T cells. On the contrary, synovial fluid from patients with active RA is predominantly infiltrated with neutrophils. All these cells are involved in the pathogenesis of RA (Firestein, 1997). However, neutrophils have the greatest potential to inflict the damage. Neutrophils play an active role in cartilage destruction and erosion secreting enzymes like collagenase, cathepsin, gelatinase, elastase and phospholipase (Edwards and Hallet, 1997). In addition, they may generate nitric oxide (NO), a series of reactive oxygen intermediates, including HOCl, and proinflammatory cytokines- tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8). All these mediators can act synergistically to accelerate inflammation and a joint damage (Edwards and Hallet, 1997).

Collagen-induced arthritis (CIA) represents one of the most established, widely used and extensively characterized animal model of RA and has been extensively used to elucidate pathogenic mechanisms relevant to human RA and also to identify potential targets for therapeutic intervention (Trentham *et al.* 1977). CIA in rat is a widely studied animal model of inflammatory polyarthritis with similarities to RA. The disease is characterized by severe swelling and erythema associated with an early massive, predominantly neutrophil cell (PMN) infiltration into the synovium (Weissmann, 1982; Edwards and Hallet, 1997). The immune response to CII involves cellular and humoral mechanisms (Klareskog *et al.* 1983). The rat model offers several advantages over the mouse model because rat is more susceptible to induction with autologous CII, which results in a more pronounced chronic relapsing disease. Intradermal injection in rats with native autologous rat collagen emulsified in IFA leads to a severe, erosive poly-arthritis suddenly developing within 2–3 weeks after immunization followed by a subsequent chronic relapsing phase (Holmdahl *et al.* 1992, 1994). The disease is genetically controlled both by MHC genes and other genes (Holmdahl *et al.* 1992; Lorentzen and Klareskog, 1996). Another hallmark of CIA model is the strong B-cell response specific for triple helical epitopes (Holmdahl *et al.* 1994; Wernhoff *et al.* 2001). These B cells are autoreactive and produce arthritogenic antibodies

(Stuart *et al.* 1982, 1983). Thus, the disease involves activation of both T and B cells that are antigen-specific and autoreactive. An unresolved issue, however, is the relative importance of T and B cells during the effector phase of the disease. Cooperation between T and B cells in the effector phase has been suggested by combined transfer experiments (Taurog *et al.* 1985; Seki *et al.* 1988). The identification of autoreactive CII-specific T cells has not yet been achieved and it is likely that these cells are tolerized but still arthritogenic as has been suggested in the mouse CIA model (Malmstrom *et al.* 1996). The development of arthritis is obviously a very complex process that can occur through different inflammatory mechanisms and that varies between strains and species. It is clear, however, that a significant portion of the inflammatory attack on the joints is mediated by pathogenic antibodies (Stuart *et al.* 1982, 1983; Kerwar *et al.* 1981; Takagishi *et al.* 1985). A role for autoreactive T cells in CIA, induced by immunization with autologous CII, is suggested by the finding that arthritis is reduced by treatment with antibodies to the T cell receptor (abTCR) at the onset of the disease, i.e. after the establishment of the antibody response (Goldschmidt and Holmdahl, 1991). Passive transfer of the disease by CII reactive T cells, however, is not as effective as in other autoimmune models such as experimental allergic encephalomyelitis (Ben-Nun *et al.* 1981) or as in adjuvant type of arthritis models (Taurog *et al.* 1983).

Disease-modifying antirheumatic drugs (DMARDs) and nonsteroidal anti-inflammatory drugs (NSAIDS) are the primary drugs used in the treatment of RA. In most of the cases, these drugs have been proved to be of only limited value as they often suppress the symptoms, but accelerate factors that promote the disease. Moreover, patients frequently become unable to continue long-term treatment with these agents due to toxicity and/or loss of benefit. Currently available DMARDs are hydroxychloroquine, sulphasalazine, methotrexate, azathioprine, leflunomide, d-penicillamine, intramuscular gold, cyclosporine and cyclophosphamide. Methotrexate (MTX) is the first-line therapy for RA. It is not only effective against inflammatory symptoms but also in the prevention or reduction of bone erosions. It is also required to achieve maximal suppression of bone destruction during the treatment of RA patients with TNF-alpha-inhibitor (Finckh *et al.* 2006), but having some side effect as well, like, cardiovascular risk factors for instance, dyslipidemias, homocysteinemia due to treatment with MTX or sulphasalazine, hypertension and renal

disease (Blom and van Riel, 2006). Disease duration has been associated with a higher risk for cardiovascular disease. Despite increased use of these combination therapies; new treatments for active RA are clearly needed.

Spirulina platensis is a filamentous cyanobacterium which grows particularly in alkaline, brackish and saline waters. *Spirulina* has been used as food and nutritional supplements for a long time (Dillon *et al.* 1995). It is also used to derive additives in pharmaceuticals and foods. It is generally regarded as a rich source of proteins, vitamins, essential amino acids, minerals, essential fatty acids such as γ-linolenic acid and sulfolipids (Campanella *et al.* 1999; Mendes *et al.* 2003). Moreover, in addition to ω-3 polyunsaturated fatty acids (PUFA), *Spirulina* has also ω-6 PUFA, phycocyanin, and other phytochemicals (Chamorro *et al.* 2002). *Spirulina* also contains phenolic acids, tocopherols and beta-carotene that are known to exhibit antioxidant properties.

Spirulina has been found to have many additional pharmacological properties. It also exhibits several potential activities like: antiviral (Hernandez-Corona *et al.* 2002), anti-arhthritic (Kumar *et al.* 2009), antiplatelet (Hsiao *et al.* 2005), anti-cardiotoxic (Khan *et al.* 2005a), hypocholesterolemic (Nagaoka *et al.* 2005), anti-nephrotoxic (Khan *et al.* 2006), and anti-hepatoxic (Mohan *et al.* 2006) effects. In other studies, *Spirulina* has been shown to prevent cataracts (Haque *et al.* 2005), acute allergic rhinitis (Mao *et al.* 2005), cerebral ischemia (Khan *et al.* 2005b; Wang *et al.* 2005), vascular reactivity (Mascher *et al.* 2005) and experimental Parkinson's (Chamorro *et al.* 2006). *Spirulina* has also been shown to be effective against cadmium (Jeyaprakash and Chinnaswamy, 2005), and $_{D}$-Ga1N- and APAP-induced liver injuries. Several of these activities are attributed to *Spirulina* itself or to some of its components including fatty acids omega-3 or omega-6, beta-carotene, alpha-tocopherol, phycocyanin, phenol compounds, and a recently isolated complex, Ca-spirulan (Ca-SP). Phycocyanin is one of the major pigment constituents of *Spirulina*, used in many countries as dietary supplement whose nutritional and therapeutic values have been very well documented. Phycocyanin has been recently reported to exibit a variety of pharmacological properties. Antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects of *Spirulina* have been experimentally attributed to phycocyanin. It was evaluated as an antioxidant *in vitro* as it was able to scavenge alkoxyl, hydroxyl and peroxyl radicals and to react with peroxinitrite and hypochlorous acid. Phycocyanin also

inhibits microsomal lipid peroxidation induced by Fe^{+2} ascorbic acid or the free radical initiator 2, 2' azobis (2-amidinopropane) hydrochloride (AAPH). Furthermore, it reduces carbon tetrachloride (CCl_4)-induced lipid peroxidation *in vivo*. Phycocyanin has been evaluated in twelve experimental models of inflammation and exerted anti-inflammatory effects in a dose dependent fashion in all of these. Thus, phycocyanin reduced edema, histamine release, myeloperoxidase (MPO) activity and the levels of prostaglandin (PGE_2) and leukotriene (LTB_4) in the inflamed tissue. These anti-inflammatory effects of phycocyanin can be due to its scavenging properties toward reactive oxygen species (ROS) and its inhibitory effects on cyclooxygenase-2 (COX-2) activity and on histamine release from mast cells. Phycocyanin also reduced the levels of TNF-α in the blood serum of mice treated with endotoxin and showed neuroprotective effects in rat cerebellar granule cell cultures and in kainite-induced brain injury in rats (Romay *et al.* 2003).

In this study we have attempted to evaluate the therapeutic efficacy of dietary *S. platensis* against CIA in rats. In this study we have characterized the physiological, histological, radiological, biochemical and immunological parameters to evaluate the effect of dietary *S. platensis* on CIA in rats. The multifunctional approach which we have used in this study would be certainly helpful in understanding the mechanism of action of *Spirulina* in the treatment of active RA and would significantly contribute to improve the pharmaceutical potential of *Spirulina.*

Objectives

The main objectives of the projects are:

1. Evaluation of anti-athritic property of dietary *S. platensis* by histopathological study in experimental arthritis.
2. Evaluation of the long term effect of dietary *S. platensis* on humoral response of experimental arthritis by ELISA against collagen type II.
3. Evaluation of the long-term effect of dietary *S. platensis* on cellular response of experimental arthritis by cytokine assay.
4. Evaluation of the long term effect of dietary *S. platensis* on cellular response of experimental arthritis by DTH reaction.
5. Evaluation of the long term effect of dietary *S. platensis* on cellular response of experimental arthritis by cell proliferation assay.

Review of Literature

2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that mainly targets the synovial tissue, cartilage and subchondral bone. It is the most common form of inflammatory arthritis, and has a substantial societal effect in terms of cost, disability, and lost productivity. Although the pathogenesis of RA remains unclear, much insight into the cellular and molecular mechanisms involved has been gained in the past decade. The continuing elucidation of pathophysiological pathways relevant in RA, coupled with continuing advances in biotechnology and rational drug design, offer substantial hope for the continued development of increasingly potent and specific pharmacotherapy for treatment of RA. On the basis of these insights, new therapies have been developed, and clinical trials have shown the efficacy of aggressive treatment of patients with active disease.

In RA, the synovial lining of diarthrodial joints is the target of an intense immunologic and inflammatory process that is associated with the proliferation of the synovial lining cells and infiltration of the tissue by inflammatory cells, including lymphocytes, plasma cells and activated macrophages. According to Drexler *et al.* (2008) numerous cellular mechanisms and signalling pathways drive the chronic inflammation in RA, and current evidence suggests an involvement of the innate as well as the adaptive immune systems in RA pathology. The importance of the adaptive immune response is supported by rodent models of disease, such as collagen-induced arthritis (CIA), that are mainly Th1- and/or Th17-driven (Cho *et al.* 2007*).* Mice lacking IL-23 do not develop CIA (Murphy *et al.* 2003). In humans, the efficacy of anti-CD20 (Rituximab) and anti-CTLA4 (Abatacept) antibodies in RA treatment suggest a function for activated B and T cells in RA (Kremer *et al.* 2003). Moreover, a role for $CD4^+$ T cells in RA pathogenesis is inferred by the strong HLA-DR association (Seldin *et al.* 1999).

Local aggregates of inflammatory cells, including macrophages and lymphocytes are often detected in the marrow space. It has been suggested that these cells are derived from the synovial lining and that they migrate into the marrow where they release local products that affect bone remodeling (Bromley and Woolley, 1984). Decreased joint motion and immobilization in response to the joint inflammation likely represent additional contributing

factors to this local bone loss. The third form of bone disease associated with RA is the presence of generalized axial and appendicular osteopenia at sites that are distant from inflamed joints (Joffe and Epstein, 1991; Peel *et al.* 1991; Woolf, 1991). Although there are conflicting reports concerning the effects of RA on skeletal mass, the presence of a generalized reduction in bone mass has been confirmed using multiple different techniques and there is compelling evidence that this reduction is associated with an increased risk of hip and vertebral fracture (Saag *et al.* 1994; Spector *et al.* 1993; Beat *et al.* 1991). The conflicting reports are in part related to the fact that most observations have been based on cross-sectional studies and have focused on patients late in the evolution of their disease when factors such as disability, corticosteroid and other treatments may confound the analyses. Compston *et al.* (1994) stated that histomorphometric analysis of bone biopsies from patients with RA indicate that, in the absence of corticosteroid use, the cellular basis of the generalized reduction in bone mass is related to a decrease in bone formation rather than an increase in bone resorption.

2.1.1 Epidemiology of RA

RA has the worldwide distribution and affects about 1% of the world population with all ethnic groups. The female: male ratio susceptible to RA is 3:1. The disease can occur at any age, but it is most common among those aged 40–70 years and its incidence increases with age (Alamanos *et al.* 2006). The geographic distribution of RA is worldwide, with a notably low prevalence in rural Africa and high prevalence in specific tribes of native Americans. However, there is no clear association between prevalence of RA and socioeconomic status. The presence of definite RA in adult Caucasians has been estimated at around 1% (Wolfe, 1968). Accurate epidemiological data are scanty in the Indian sub-continent and South-East Asian countries but the prevalence has been variously estimated at between 0.2 and 1.1% (Malaviya *et al.* 1993; Darmawan *et al.* 1993; De *et al.* 1993). From the available data in India, the prevalence of RA seems to be about the same as in Caucasians with no definite evidence of a lower prevalence in rural areas (Malaviya *et al.* 1983) as seems to be the case in Africa (McGill, 1991). In Indonesia, the age-adjusted prevalence rate of RA is 25% in urban and rural areas (Darmawan *et al.* 1993). In southern Indians, the age of onset of RA and sex ratio is similar to Caucasians, although the number of elderly males with RA is recorded more. Incidence data on RA have been collected mostly in populations of Anglo-

Saxon origin. The incidence of RA ranges from around 20-300 per 100,000 adults per year. The available data on RA prevalence derive particularly from studies performed in the USA and Europe, with minimal information on other parts of the world. The prevalence of RA in most industrialized countries varies between 0.3 and 1% and a reasonable overall prevalence for definite RA is of 0.8% adults having age of 15^{+}. The prevalence of RA in developing countries is variable; with some studies showing lower prevalence rates and others having similar levels to those in developed countries (Abdel-Nasser *et al.* 1997). Kinne *et al.* (2007) stated that females are affected by RA at a ratio of approximately 3:1 compared with males and experience clinical fluctuations during the menstrual cycle and pregnancy, indicating a major modulating role for sex hormones. Due to their expression of sex-hormone receptors and their cytokine response upon exposure to estrogens, monocytes/macrophages are strongly involved in hormone modulation of RA (Cutolo and Lahita, 2005). Indeed, physiological levels of estrogens stimulate RA macrophages to the production of the pro-inflammatory cytokine IL-1, whereas, higher levels inhibit IL-1 production, conceivably mimicking the clinical improvement during pregnancy. Interestingly, selective estrogen receptor ligands inhibiting nuclear factor (NF)-κB transcriptional activity (but lacking estrogenic activity) can markedly inhibit joint swelling and destruction in experimental arthritis (Keith *et al.* 2005). RA affects 0.5-1% of the adult population (Malaviya *et al.* 1993; Chopra *et al.* 2001) as well.

2.1.2 Role of cytokines in RA

Cytokines are small soluble proteins that mediate intercellular communication between cells involved in immune responses, affect cell division, differentiation, and chemotaxis, as well as more broadly defined pro-inflammatory or anti-inflammatory actions. Quantitative analyses suggest that there are few T-cell derived cytokines (such as IL-2 and IL-17, and IFN-γ) in inflamed synovial tissue; however, many other cytokines are present in moderate to high concentrations in RA. TNF-α and IL-1 are both present in large quantities in affected synovial fluid, serum and synovial tissue. Immunohistochemical and mRNA *in-situ* hybridization analysis have shown the presence of these cytokines in cells of the synovial lining and sublining, including type-A synoviocytes and other macrophage like populations (Wood *et al.* 1992a; Chu *et al.* 1991). Both TNF-α and IL-1 are potent *in-vitro* stimulators of synovial tissue effector functions including proliferation, metalloproteinase expression,

adhesion-molecule expression, secretion of other cytokines, and prostaglandin production. TNF-α and IL-1 seem to function synergistically in inducing effector function. To provide a means for homoeostasis and down regulation of inflammatory responses, a subclass of cytokines and cytokine receptors are thought to exert anti-inflammatory activity in the synovium. There are two TNF receptors (p55 and p75), both of which occur naturally in synovial fluid in soluble form, inhibiting TNF-α activity by competing with cell-surface receptors for binding. Additionally, a naturally occurring competitive inhibitor for IL-1 at the IL-1 receptor (IL-1 receptor antagonist) is also present in RA synovial fluid (Firestein *et al*. 1992). This member of the IL-1 family binds to IL-1R1 without transducing a signal, thus blocking the receptor binding ability of IL-1α or IL-1β. Results of work in animals have suggested a central role for TNF-α and IL-1 in the process of synovitis and joint destruction. Addition of exogenous IL-1 or TNF-α into experimental models of arthritis induces or exacerbates synovitis. Furthermore, mice transgenic for TNF-α, and mice with dysregulated TNF-α production develop arthritis (Taylor *et al.* 1996). Treatment of murine models of arthritis with antibodies against TNF-α or IL-1 or with soluble TNF-α receptor ameliorates or abrogates disease (Taylor *et al.* 1996; Williams *et al*. 1992; Joosten *et al*. 1996). IL-10, a powerful anti-inflammatory cytokine is able to suppress the production of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1 from macrophages. Its role in RA disease-associated macrophages, however, is controversial. Human IL-10 has little effect when used to alleviate disease in RA patients. IL-10 has also been shown to up regulate various genes associated with pro-inflammatory function, as well as the IFN-γ-inducible genes. In response to IL-10, RA macrophages up regulate TNF receptor (TNFR) 1 and TNFR2 mRNA and produce elevated levels of IL-1β and IL-6 in response to TNF-α and macrophage-colony stimulating factor (Takasugi *et al.* 2006). In sharp contrast to the RA macrophage phenotype, IL-10 treatment of CIA mice has also been shown to inhibit disease progression (Saidenberg-Kermanac'h *et al.* 2003). Overall, this suggests that arthritic macrophages may have altered signaling patterns when compared to other cell types, and IL-10 may have both anti- and pro-inflammatory functions in RA.

2.1.3 Experimental model of RA

Experimental animal models of arthritis provide the possibility to investigate pathogenesis, diagnosis and treatment of arthritis which is many aspects is comparable with human's RA.

Animal models may still differ from the human disease in some aspects. Nevertheless, they offer the possibility of investigating disease mechanisms and new potential protective as well as therapeutic treatments which otherwise could not be evaluated in human due to reasonable ethical reasons.

2.2 Collagen induced arthritis

Collagen induced arthritis (CIA) is one of the most established animal model for RA that is induced intradermaly in susceptible rat or mouse strains with collagen-II (CII) emulsified in an adjuvant. CII is a major protein constituent of joint cartilage and the immunization provokes an autoimmune response that attacks the joints. The two arms of adaptive immunity, T and B cells play a central role in the pathogenesis of CIA but their relative importance in both priming of immune activation and joint destruction are still unclear. The major role of B cells is production of arthritogenic anti-CII antibodies, which is clearly shown by the fact that antibodies reactive with CII can bind to cartilage and induce arthritis (Terato *et al.* 1992). Moreover, B cell-deficient mice are resistant to CIA (Svensson *et al.* 1998). The role of T cells in CIA is more complex and can be divided into two main pathways, which synergize in the development of arthritis. Firstly, T cells provide help to B cells for the production of arthritogenic anti-CII antibodies (Corthay *et al.* 1999). Secondly, T cells themselves are believed to play a role in joint inflammation through activation of other cells, e.g. synovial macrophages. Transfer of T cells can induce synovitis but not clinically evident arthritis, and blockage of T cells or T cell function ameliorates the development of arthritis (Goldschmidt and Holmdahl, 1991; Holmdahl *et al.* 1985b; Taylor *et al.* 1996; Williams *et al.* 1992).

2.2.1 Characteristics of the CIA model

CIA is most efficiently induced after a single immunization with heterologous CII, i.e. CII derived from a species other than the mouse (Holmdahl *et al.* 1990). This results in immune priming after a few days, immune activation in the joints after 1 or 2 weeks and a sudden onset of macroscopic arthritis not earlier than 2 weeks, and in most cases several weeks or months after immunization. Within a few days after arthritis onset, the affected joint is damaged by an inflammatory reaction where the severe destruction of the joint has a very similar histopathology as observed in RA with formation of an erosive pannus tissue and remodeling of the joint. The active inflammation will subside within 3–4 weeks after disease

onset leaving a destroyed and remodeled, but inflammation-free joint. However, a recent discovery is that in some strains a chronic relapsing disease may reappear months later. Most mouse strains are more resistant to development of arthritis after immunization with autologous (mouse) CII. However, if arthritis develops, it is usually severe and will progress into a chronic relapsing disease earlier than is normally seen after immunization with heterologous CII (Holmdahl *et al.* 1986; Malfait *et al.* 2001). For the induction of arthritis, a strong immune response to CII is of importance. This is achieved by immunization with CII in adjuvant, usually mineral oil with mycobacterial cell walls (complete Freund's adjuvant (CFA)). This will induce a typical type 1 response with a strong IL-12 and IFN production as well as the secretion of immunoglobulins of all isotypes. However, in certain strains arthritis will also develop after immunization with CII in mineral oil only, so called incomplete Freund's adjuvant (IFA). In this case the immune response is slightly shifted towards a type 2 response with a dominance of the IgG1 isotype in the antibody response. Thus, both type 1 and type 2 responses seem to contribute to the development of arthritis.

2.2.2 Histological changes during CIA

Histological changes are characterized by symmetrical, erosive synovitis, and in some cases, extra-articular involvement. An inflamed synovium is central to the pathophysiology of RA. It is histologically striking, showing pronounced angiogenesis; cellular hyperplasia; an influx of inflammatory leucocytes; and changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors, and many cytokines. Synovial changes in RA vary with disease progression. In the first weeks of the disease, tissue edema and fibrin deposition are prominent and can manifest clinically as joint swelling and pain. Within a short period, the synovial lining becomes hyperplastic, commonly becoming ten or more cells deep and consisting of type A (macrophage-like) and type B (fibroblast-like) synoviocytes. The sublining also undergoes striking alterations in cellular number and content, with prominent infiltration of mononuclear cells including T cells, B cells, macrophages, and plasma cells. Synovial-vessel endothelial cells transform into high endothelial venules early in the course of the disease (Girard *et al.* 1995). The formation of locally invasive synovial tissue pannus is a characteristic feature of RA. This tissue is involved in the joint erosions in RA. Pannus is histologically distinct from other regions of

the synovium and shows phases of progression. Initially, there is penetration of the cartilage by synovial pannus composed of mononuclear cells and fibroblasts (Shiozawa *et al.* 1983) with high-level expression of matrix metalloproteinases by synovial lining cells (McCachren *et al.* 1990; Gravallese *et al.* 1991). In later phases of the disease, cellular pannus can be replaced by fibrous pannus comprised of a minimally vascularised layer of pannus cells and collagen overlying cartilage (Kobayashi and Ziff, 1975). However, the molecular pathogenic mechanisms driving pannus formation remain poorly understood. The tissue derivation of pannus cells has not been fully elucidated, although they are thought to arise from fibroblast-like cells (type B synoviocytes). *In-vitro* experiment shows that these fibroblast-like synoviocytes have anchorage-independent proliferation and loss of contact inhibition (Lafyatis *et al.* 1989), which are phenotypes shown by transformed cells. *In vitro* studies in chondrocytes have elucidated signaling pathways and transcription factors that orchestrate specific functions that promote cartilage damage in both OA and RA. Thus, understanding how the adult articular chondrocyte functions within its unique environment will aid in the development of rational strategies to protect cartilage from damage resulting from joint disease (Goldring and Marcu, 2009).

2.3 RA therapy

The degree of effectiveness of therapeutic treatments in clinical practice in the community, however, has not been established. Health status and functional status are central components of RA outcomes. In the fifties and early sixties, the treatment of RA revolved the use of high-dose aspirin, other non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. These drugs provided symptomatic relief but did not have any significant effect on the underlying disease process. In addition, they caused a fair degree of side effects. Subsequently, diseases modifying antirheumatic drugs (DMARDs) were added to the existing armamentarium. Others include gold, d-penicillamine, chloroquine and hydroxychloroquine. These drugs had their limitations. Sulphasalazine was designed for the treatment of RA but failed in its initial trials to produce benefit. The drug was rediscovered about 30 years later and became an important addition to the other DMARDs. Methotrexate, sulfasalazine, hydroxychloroquine and cyclosporine, either alone, or in combination, have been the principal therapies for RA in the last decade (Kremer, 2001; Moreland, 2001; McInnes, 2001). It is now well established that early therapy with DMARDs is critical for

better long-term outcome in RA (Lard *et al.* 2001). Also, combinations of DMARDs are well tolerated and results are better than monotherapy. It is well recognized that erosive changes occur early in the disease, often in the first year (Van der, 1995). In addition, mortality among RA patients who present early is lower than those who present later in the course of the disease (Symmons *et al.* 1998). Early and aggressive therapy is the current standard of care for RA. The last 4 years or so have seen the advent of several new therapeutic agents for RA. Firstly, safer NSAIDs or 'Coxibs' have become available. The list, which began with celecoxib seems to be expanding fast with the addition of rofecoxib, valdecoxib and etoricoxib etc. Secondly, interventions designed specifically to target pathogenic cytokines have reached the clinic. These include most notably, anti-TNF antibody or 'infliximab', soluble TNF-α receptor or 'etanercept' and IL-1 receptor antagonist or 'anakinra'. The other very important new agent is leflunomide. Specific Cox-2 inhibitors were introduced into clinical practice recently. They reduce the incidence of serious gastro-intestinal events by about 80% of that observed with conventional NSAIDs. Patients likely to benefit most with Cox-2 inhibitors include elderly, those taking steroids or anticoagulants and those with past history of peptic ulcers. However, these agents lack antiplatelet effect. This has important implications in the context of RA. At present there is uncertainty about the efficacy of even conventional NSAIDs as substitutes for low dose aspirin in coronary artery disease (CAD) prophylaxis. Patients taking low dose aspirin for CAD or stroke must not discontinue it. Leflunomide is an oral antimetabolite, which has shown efficacy in the treatment of RA. It is a pro-drug which is rapidly converted to its active form, which inhibits dihydro-orotate dehydrogenase, the rate-limiting enzyme for *de novo* synthesis of pyrimidine nucleotides. Currently, the drug merits the status of a very good alternative to methotrexate. Combination of leflunomide with methotrexate has been found to be superior to methotrexate alone. The side effects of the drug in general include nausea, diarrhoea, drug rash, reversible alopecia, transient mild transaminitis and hypertension. It is advisable to monitor the blood pressure and liver functions periodically. Leflunomide is teratogenic and thus contraindicated in pregnant women and those contemplating pregnancy. Similarly, it is also not recommended in men who wish to have further children. Advances in the pathogenesis of RA have led to the use of a number of biological agents in the last 2 decades. Recognition of the central role of pro-inflammatory

cytokines (TNF-α and IL-1) culminated in the successful use of TNF-α antagonists and IL-1 receptor antagonist for the treatment of RA. However, the present cost of these drugs is prohibitive, especially, for developing countries. Etanercept was the first of these agents introduced for the treatment of RA. It consists of high affinity type II TNF-α receptors covalently linked to the Fc portion of IgG1. Etanercept has high affinity for TNF-α. The other TNF antagonist is infliximab. It is a chimeric mouse/human anti-TNF-α monoclonal antibody (mAb) developed using recombinant technologies. It binds and inactivates soluble TNF-α. Human IL-1 Ra (Anakinra) is a cloned replica of the human cytokine from which it is derived. All the three agents have been shown to be effective in management of RA refractory to multiple DMARDs. Methotrexate is usually combined with these agents (Kavanaugh *et al.* 2000).

Since in clinical trials maximal therapeutic responsiveness can be seen within 3 to 6 months and since disease activity at 3 to 6 months is an excellent predictor of activity at 12 months (Aletaha *et al.* 2007), all necessary decisions can be made at that time, for the sake of the patient and consequently for society.

2.4 *Spirulina*

Spirulina is a photosynthetic, filamentous, spiral-shaped, multicellular blue- green microalga. *Spirulina* used for the production of nutritional supplements is either grown in outdoor tanks or harvested from lakes as grown in Mexico, Central and South America, and Africa. *Spirulina*, technically not an herb, that was gathered by the ancient Aztecs, other native American tribes and members of certain African tribes from alkaline lakes and ponds where it grew in abundance. It was eaten as a nourishing food concentrate. Research has proved its high nutritive value and has lent credence to the claim of *Spirulina* as a high-energy super food and possible appetite suppressant. Dietary excesses and cravings can often be controlled by adding *Spirulina* to the diet. Lu J *et al.* (2010) correlates well across several mammalian species with several parameters of biology including energy expenditure and basal metabolism, the effective dose of *Spirulina* (6%) would be about 42 g for an average person (60 kg) per day.

2.4.1 Anti-inflammatory effects of *Spirulina*

The first report on antioxidant and anti-inflammatory properties of c-phycocyanin from *Spirulina* (Romay *et al*. 1998a) showed that phycocyanin was able to scavenge hydroxyl

(IC_{50} = 0.91 mg ml^{-1}) and alkoxyl (IC_{50}= 76 μg ml^{-1}) radicals with activity equal to 0.125 mg $ml^{-1}$of dimethyl sulfoxide (DMSO) and 0.038 μg $ml^{-1}$of trolox, specific scavengers of those radicals, respectively. Phycocyanin also inhibited liver microsomal lipid peroxidation (IC_{50}=12 mg ml^{-1}). The oxygen-scavenging activity of c-phycocyanin was only 3 times lower than that of superoxide dismutase (SOD). The addition of SOD to the phycocyanin did not alter the antioxidant activity of the phycocyanin, suggesting a different mechanism of action (Romay *et al*. 1998a). Further studies by the same group (Romay *et al*. 1998b) revealed the anti-inflammatory activity of phycocyanin in some animal models of inflammation. Phycocyanin reduced significantly and in a dose dependent manner the ear edema induced by arachidonic acid and tissue plasminogen activator in mice as well as carageenan induced rat paw edema. Phycocyanin also showed anti-inflammatory activity in a subchronic cotton pellet granuloma test where sterile cotton pellets were implanted in the axillae of rats. Oral administration of phycocyanin resulted in significant anti-inflammatory activity in all models tested. The anti-inflammatory activity observed was attributed to antioxidant and oxygen scavenging activity of phycocyanin and perhaps also due to its inhibitory effect on arachidonic acid metabolism. When compared with indomethacin, a standard anti-inflammatory drug, phycocyanin showed a weaker activity (50-300 mg kg^{-1}, p.o.) as compared to 3-10 mg kg^{-1}, p.o., for the former. However, the LD_{50} of indomethacin was 12 mg kg^{-1}in rats and 50 mg kg^{-1} in mice, p.o., and induces many side effects in patients under treatment. The LD_{50} of phycocyanin in rats and mice was greater than 3g kg^{-1}, p.o. In fact, no mortality was observed even at 3 g kg^{-1}, p.o. (Romay *et al*. 1998b). Vadiraja *et al.* (1998) studied the effect of c-phycocyanin from *S. platensis* on carbon tetrachloride and R-(+)-pulegone-induced hepatotoxicity in rats. In this study a single dose (200 mg kg^{-1}) of phycocyanin was administered intraperitoneally to rats one or three hours prior to R-(+)-pulegone (250 mg kg^{-1}) or carbon tetrachloride (0.6 ml kg^{-1}) challenge. The fact that orally administered phycocyanin or *Spirulina* exerts potent and versatile anti-inflammatory effects in rodents (Romay *et al.* 2003; Rimbau *et al.* 1999; Chamorro *et al.* 2006; Remirez *et al.* 2002; Rasool *et al.* 2006; Khan *et al.* 2006; Mohan *et al.* 2006) strongly suggests that ingested phycocyanin can be sufficiently well absorbed to provide important systemic antioxidant activity. Phycocyanin administered orally to mice and rats exerts a number of dose-dependent anti-inflammatory effects in a dose range of 50–300 mg $kg^{-1}day^{-1}$ (Romay *et*

al. 2003; Rimbau *et al.* 1999). Recent studies in which whole *Spirulina* has been administered orally to rodents have also shown anti-inflammatory effects, in doses ranging from 150 to 1,000 mg $kg^{-1}day^{-1}$ (Chamorro *et al.* 2006; Remirez *et al.* 2002; Rasool *et al.* 2006; Khan *et al.* 2006; Mohan *et al.* 2006). The evidence for anti-inflammatory effect of *Spirulina platensis* was give by Kumar *et al.* (2009) in female wistar rats.

2.4.2 Antioxidant effects of *Spirulina*

The antioxidant properties of *Spirulina* and its extracts have attracted the attention of researchers recently. Manoj *et al*. (1992) have reported that the alcohol extract of *Spirulina* inhibited lipid peroxidation more significantly (65% inhibition) than the chemical antioxidants like α-tocopherol (35%), and β-carotene (48%). The water extract of *Spirulina* was also shown to have more antioxidant effect (76%) than gallic acid (54%) and chlorogenic acid (56%). An interesting aspect of this finding is that the water extract had a significant antioxidant effect even after the removal of polyphenols. Zhi-gang *et al*. (1997) have studied the antioxidant effects of two fractions of a hot water extract of *Spirulina* using three systems that generate superoxide, lipid, and hydroxyl radicals. Both the fractions showed significant capacity to scavenge hydroxyl radicals (the most highly reactive oxygen radical) but no effect on superoxide radicals. One fraction had significant activity in scavenging lipid radicals at low concentrations. In a study by Miranda *et al.* (1998), the antioxidant activity of a methanolic extract of *Spirulina* was determined *in vitro* and *in vivo*. The *in vitro* antioxidant assay involved a brain homogenate incubated with and without the extract at 37^0C. Peroxidation of rat brain homogenate was inhibited by almost 95% with 0.5 mg of the methanolic extract. The IC_{50} of the extract in this system was found to be 180 μg. The *in vivo* antioxidant capacity was evaluated in plasma and liver of animals receiving a daily dose of 5 mg for 2 and 7 weeks. Plasma antioxidant activity in brain homogenate incubated at 47^0C showed that the antioxidant capacity of plasma was 97 and 71% for the experimental group and 74 and 54% for the control group after 2 and 7 months, respectively. The antioxidant effect of *Spirulina* was attributed to beta-carotene, tocopherol, and phenolic compounds working individually or in synergy (Miranda *et al.* 1998).

The c-phycocyanin from *S. platensis* is a selective inhibitor of cyclooxygenase –2 (COX-2) with very low IC_{50} COX-2/IC_{50} COX-1 ratio (0.04) (Reddy *et al.* 2000). Interestingly, this study showed that the IC_{50} value obtained for COX-2 inhibition by

phycocyanin was much lower (180 nM) as compared to those for celecoxib (255 nM) and rofecoxib (401 nM), the well-known selective COX-2 inhibitors. The apoprotein component of phycocyanin was responsible for the inhibition of COX-2 since reduced phycocyanin and phycocyanobilin were found to be ineffective. It has been suggested that the hepatoprotective, anti-inflammatory, and anti-arthritic properties of phycocyanin reported might be due, in part, to its selective COX-2 inhibitory property, though a similar effect of phycocyanin was not excluded through its ability to efficiently scavenge free radicals and inhibit lipid peroxidation. *Spirulina* also has a significant effect on free radical scavenging (Upasani and Balaraman, 2003). Some of the pharmacological activities may be attributed to the antioxidant activity of *Spirulina* (Lu *et al.* 2006), or *Spirulina* extracts (Pinero-Estrada *et al.* 2001; Wu *et al.* 2005) or of some of its components such as phycocyanin (Hsiao *et al.* 2005; Nagaoka *et al.* 2005).

Materials and methods

This chapter contains the details of materials and methods employed throughout the course of this research work.

3.1 Reagents

All chemicals (inorganic and organic) used were of analytical grade and unless otherwise stated were purchased from the Sigma Chemical Co., St. Louis, Missouri (USA) and BDH, Poole, England. Water used in the preparation of media and solutions was double glass-distilled.

3.2 Sterilization

Growth medium used for the mass cultivation of *Spirulina platensis* was steam sterilized in an autoclave at 121^0C and at a pressure of 15 lbs $inch^{-2}$ for 15 min. All the glassware were sterilized in a hot air oven at 160^0C for 2 h before being used. The chemicals which are heat-labile and may be affected by high temperature during autoclaving were sterilized by filtration through Millipore filters.

3.3 *Spirulina platensis* and therapeutic drug

S. platensis was obtained from National Centre for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute, New Delhi, India. The Methotrexate tablets used were procured from Emil Pharmaceutical Industries Pvt. Ltd. N-50, MIDC, Tarapur, Thane, Maharashtra, India.

3.4 Mass cultivation of *S. platensis*

S. platensis was axenically grown in Zarrouk's medium (Zarrouk, 1966) whose composition is given in Table 1. The cultures were incubated in a culture room at 25^0C and illuminated with day-light fluorescent tubes having the photon fluence rate of 50 μmol m^{-2} s^{-1} on the surface of the vessels.

Table 1: Composition of Zarrouk's medium

Macroelements	**g l^{-1}**
$CaCl_2$ $2H_2O$	0.04
NaCl	1.0
$NaNO_3$	2.5
$FeSO_4$ $7H_2O$	0.01
EDTA	0.08
K_2SO_4	1.0
$MgSO_4$ $7H_2O$	0.2
$NaHCO_3$	16.8
K_2HPO_4	0.5

Micro elements	***g l^{-1}***
$ZnSO_4$ $7H_2O$	0.22
$CuSO_4$ $5H_2O$	0.79
MoO_3	0.015
H_3BO_3	2.86
$MnCl_2$ $4H_2O$	1.81

For preparing 1 liter Zarrouk's medium, macroelements were weighted and dissolved in one liter distilled water. Micronutrients stock was prepared separately in 1 liter distilled water. To 1 liter macroelements solution, 1 ml of micronutrients stock was added and the content was mixed thoroughly. The pH of the medium was adjusted to 8.5-9 before autoclaving.

3.5 Biomass preparation of *S. platensis*

Harvesting of *S. platensis* biomass is one of the most crucial steps in culturing of the *S. platensis*. Effective harvesting is required to preserve the quality and also to improve the culture process. For the preparation of dried biomass of *S. platensis*, exponentially growing cells of S. *platensis* were harvested by filtration (screen-printing filter with pore size 305 nm (1,400 pore/cm^2) and the biomass was dried at 50^0C. The dried *S. platensis* biomass was collected, weighed and used to feed the experimental animals (rats).

3.6 Experimental design and animals

Albino female rats (Wistar strain) of 6-10 weeks of age, obtained from Defense Research Development Establishment (DRDE), Gwalior, India, were used as the experimental animals in the proposed study. The rats were acclimatized for a week and colony was maintained in the animal house with 12:12 h light: dark schedule and free access to food and water was given *ad libitum*.

Rats were subdivided into the following groups:

1. Normal control rats (n=6)
2. Arthritic control rats (n=6)
3. Methotrexate treated arthritic rats 0.3 mg kg^{-1} (n=6)
4. *S. platensis* treated arthritic rats 400 mg kg^{-1} (n=6)
5. *S. platensis* treated arthritic rats 800 mg kg^{-1} (n=6)

All the experimental protocols were pre-approved by the animal ethical committee, Jiwaji University, Gwalior, Madhya Pradesh (India).

3.7 Antigen preparation

Collagen from bovine tracheal cartilage type II obtained from Sigma chemical company St. Louis, Missouri, USA (CII) was dissolved in cold 0.1N acetic acid (2 mg ml^{-1}) and was emulsified with an equal volume of freshly opened, cold freund's adjuvant incomplete (IFA) (Sigma, USA). The emulsion was made by using three-way stopcock with syringe.

3.8 Development of collagen induced arthritis (CIA) in rats

Collagen induced arthritis (CIA) in rats was developed according to Remmers *et al.* (2002). Rats were injected intradermally at several sites on the back with a dose of 2 mg kg^{-1} of body weight. For this the hairs at the back of rat were removed by using razor blade and then immunized with 1 ml tuberculin syringe. On the seventh day after the primary immunization, the rats were boosted with 0.1 ml (100 μg) of similarly prepared collagen/ IFA emulsion injected intradermally at the base of the tail.

3.9 Treatment of animal with *S. platensis* biomass and methotrexate

The water suspension of *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) were administered orally to arthritic rats on a daily basis and once a week, respectively with the help of syringe cannula. Sterile water was, however, given to the normal control as well

as untreated arthritic control rats. The treatment of *S. platensis* and methotrexate was started from day 20th post collagen immunization up to 45th day.

3.10 Assessment of arthritis and arthritic score

Rats were screened for the development and progression of arthritis daily from day 0 to 45th day with 5 day interval.

The severity of arthritis was graded as: Grade 0 = no sign of arthritis, Grade 1 = redness and swelling in paw, Grade 2 = deformity in paw, Grade 3 = ankylosis in paw, Grade 4 = Maximal swelling and deformity with ankylosis

The arthritic score of a diseased rat was sum of the maximum grades of arthritis in the involved paws. The data were expressed as mean±SEM of six animals per group from day 0 to day 45th recorded at an interval of 5 day.

3.11 Changes paw thickness

Severity of arthritis in hind paw was assessed by the quantification of the changes in paw thickness. Measurements were made with a dial gauge caliper from 0 to 45th day at an interval of every 5 day. The data were expressed as mean±SEM of six animals per group.

3.12 Ankle size

Severity of arthritis was also assessed by quantifying the changes in ankle size. Measurement was made with a dial gauge caliper from 0 to 45th day and the data were expressed as mean ±SEM of six animals per group.

3.13 Change in body weight

The body weight of normal control, arthritic control and *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats was measured from 0 to day 45th at an interval of every 5th day and the data were expressed as mean±SEM of six animals per group.

3.14 Measurement of arthritic pain

Arthritic pain of arthritic control and *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats was evaluated using the foot print assessment of pain which reflects the spontaneous pain behavior. The rat's hind paws were dipped in ink and the animals were allowed to walk on white paper for a distance of 60 cm from day 20th to day 45th. The data are recorded at an interval of every 5 day and expressed as mean±SEM of six animals per group.

The foot prints were scored as follow: 0= normal footprint, 1= partial footprint (no heel), 2= only fingers, 3= absence of one footprint, 4= total absence of footprint

3.15 Measurement of total print length, 1-5 toe spread and 2-4 toe spread

According to Bain *et al.* (1989) and Walker *et al.* (1994) the functional recovery of normal control, arthritic control and treated arthritic rats was measured by allowing the animal to explore in a 10 cm wide and 60 cm long wooden corridor on a sheet of ink absorbing paper, with their hind paws dipped in blue ink on day 45^{th} post collagen immunization. Walking on the sheet resulted in at least three to four prints of each foot. Individual walking print length, 1-5 toe spread and 2-4 toe spread values were measured with a dial gauge caliper. The data were expressed as mean±SEM of six animals per group.

3.16 Radiography

The rats were anaesthetized with ketamin (200µl, *i.p.)* and anaesthetized rats were placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and arthritic hind paws was performed by X-ray machine (Philips X12 Germany) with a 40 kW exposition for 0.01 s.

The following radiograph criteria were considered:

0 = no bone damage, 1 = tissue swelling and oedema, 2 = joint erosion, 3 =bone erosion and osteophyte formation

3.17 Histopathological Examination

Paw joints were collected at the end of experiment (45^{th} day) and after that tissue samples of the joints (10 mm x 5 mm thick pieces) were fixed in 10% (v/v) neutral formalin. The formalin fixed tissues were cut into thin pieces (2-3 mm thick) and decalcified in 10% EDTA for 21 days. The decalcified tissue pieces were washed in running tap water for overnight and washed twice with distilled water for 30 min.

The decalcified tissues were dehydrated in ascending grades of alcohol and cleaned. Dehydration was done in 30 and 50% alcohol for 1 h followed by 70% alcohol for overnight. These tissues were further dehydrated with 90% alcohol for 2 h. This step was repeated twice with absolute alcohol for 2 h. After dehydration, the joint tissues were kept in toluene twice for 1 h each. After that the tissues were kept at 65^0C, dipped in high melting fresh paraffin for overnight in oven for paraffin impregnation and the tissues were

transferred to fresh paraffin for 5 h. Blocks were prepared in fresh paraffin. Following paraffin embedding, 7µ thick sections were cut with rotory microtome (Leica RM 2135).

After sectioning, the sections were deparaffinzed in xyline for about 1 h at 58^0C in oven. After deparaffinization, the rehydration of sections was carried out with descending grade of alcohol. Rehydration included 100, 90, 70, 50 and 30% alcohol for 10 min each. After rehydration, the sections were washed in running tap water for 2 min and then rinsed with distilled water. After rinsing, the sections were dipped 6-7 times in Harris haematoxylin followed by washing with running tap water for 5 min. Sections were dipped 4-5 times in HCl water for differentiation followed by washing with running tap water for 15 min and finally rinsed with distilled water. Sections were dehydrated in 30 and 50% alcohol for 5 min each and in 70% alcohol for 8 min, followed by counterstaining in 1% eosin stain in 90% alcohol for 10 min. Destaining of sections was done by dipping the tissues once or twice in 90% alcohol, followed by 1-2 dip in absolute alcohol, with a repeated change of absolute alcohol for 3 and 12 min. After destaining, the sections were kept in xylene for 25 min. The sections were mounted in DPX (Humason, 1972).

3.18 Biochemical parameters

It includes the determination of total cholesterol, albumin, serum protein, plasma protein, acid phosphatase, alkaline phosphatase, lipid peroxidation, and serum glutamate pyruvate transminase.

3.18.1 Sample collection and processing

The blood samples were drawn from the retro-orbital bleeding. The blood was collected in tubes containing EDTA (an anticoagulant, 2mg ml^{-1}) and without EDTA (anticoagulant) for plasma and serum, respectively. Blood samples were centrifuged for 10 min at 5,000 rpm and at 4^0C and serum and plasma collected were stored at – 20^0C for further investigation.

3.18.2 Total serum cholesterol

Serum cholesterol level was estimated by CHOD-PAP method using kit (Merck India Ltd.).

Assay procedure

One ml of reaction solution was added in each tube. To that 10 µl of serum sample was added and the mixture was incubated at room temperature for 20 min. The concentration of cholesterol was measured with 10 µl of cholesterol standard (200mg dl^{-1}) provided with the

kit. Absorbance was recorded at a wavelength of 546 nm against the buffer as a blank by PC base UV-VIS double beam spectrophotometer-2202 (Systronics).

Calculation

$$\text{Cholesterol (mg dl}^{-1}) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg dl}^{-1})$$

3.18.3 Total serum albumin

Total serum albumin was assayed according to Doumas *et al.* (1971) using kit (Merck India Ltd). Albumin forms blue-green complex with bromocresol green at slightly acidic pH, which is measured spectrophotometrically.

Assay procedure

One ml of reaction solution was added in each tube. To that 10 µl of serum sample was added, the solution was mixed well and incubated at 37^0C in water bath for 10 min. The concentration of albumin was measured with 10 µl of albumin standard (5mg dl^{-1}) provided with the kit. Absorbance was recorded at 540 nm against the buffer as a blank.

Calculation

$$\text{Serum albumin (gdl}^{-1}) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (g dl}^{-1})$$

3.18.4 Total serum protein

Total plasma protein and total serum protein in blood samples collected from normal control, arthritic control, arthritic *S. platensis* and methotrexate treated rats were measured following the method of Lowry *et al.* (1951).

Reagents

A) Alkaline Na_2CO_3 solution : 20g l^{-1} in 0.1M NaOH

B) $CuSO_4$ and Na-K tartarate solution : 5g l^{-1} $CuSO_4$ in 10g l^{-1} Na-K tartarate solution

C) Alkaline solution : 50 ml of alkaline Na_2CO_3 + 1ml of $CuSO_4$ and Na-K tartarate solution

D) Folin Ciocalteau phenol reagent : 1N

Assay procedure

To 1 ml of diluted serum sample, 5 ml of alkaline solution was added and the contents were mixed thoroughly. After 10 min of incubation at room temperature, 0.5 ml of 1N Folin phenol reagent was added and the contents were vortexed immediately. A reagent blank with 1 ml of distilled water along with the standards and sample was also prepared. After 30 min of incubation at room temperature, the absorbance of the blue color developed was recorded at 750 nm against a reagent blank using UV-VIS Spectrophotometer. Concentration of protein in the unknown samples was quantified by using a calibration curve prepared for bovine serum albumin (Sigma).

3.18.5 Serum alkaline phosphatase activity

Serum alkaline phosphatase activity was assayed according to Kind and King's (1954).

Serum alkaline phosphatase converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4- aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide and forms an orange-red colored complex which is measured spectrophotometrically. The color intensity is proportional to the enzyme activity.

Sample: Serum sample collected from rats.

Assay procedure

Test

One ml of buffer (pH 10) was added to an equal amount of substrate (M/100 disodium phenyl phosphate) and the mixture was kept at 37^0C for 3 min. To that 0.1 ml serum was added and the content was mixed. The solution was incubated at 37^0C for 15 min in a water bath. After that 0.8 ml N/2 NaOH and 1.2 ml M/2 $NaHCO_3$ were added. Then 1ml 0.6% 4-aminoantipyrine (A.A.P.) was added and the content was mixed. Finally 1 ml 2.4% $K_3Fe(CN)_6$ was added and the content was mixed thoroughly.

Control

Control was similar to test except that serum was added after NaOH.

Standard

Alkaline buffer 1.1 ml, 1 ml phenol working standard (0.01mg) and 0.8 ml N/2 NaOH were mixed together. To that 1.2 ml M/2 $NaHCO_3$ was added. After that 1ml 0.6% A.A.P. was

added and the content was mixed thoroughly. Finally 1 ml 2.4% $K_3Fe(CN)_6$ was added and the content was mixed.

Blank

Blank was similar to the standard but water was substituted for phenol.

The color developed immediately and was stable for at least an hour if kept in dim light. The absorbance of the color developed was recorded spectrophotometrically at 510 nm against a reagent blank.

Calculation

Serum alkaline phosphatase activity in KA units per 100 ml was calculated as:

$$\text{Serum alkaline phosphatase activity} = \frac{\text{O.D. Test} - \text{O.D. Control}}{\text{O.D. Standard} - \text{O.D. Blank}} \times 10$$

3.18.6 Serum acid phosphatase assay

Serum acid phosphatase activity was assayed according to King and Jagatheesan (1959). Acid phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 4.9. Phenol so formed reacts in alkaline medium with aminoantipyrine in the presence of oxidizing agent, potassium ferricyanide and forms an orange red colored complex whose intensity is measured colorimetrically. The color intensity is proportional to the enzyme activity.

Sample: Serum sample collected from rats.

Assay Procedure

Test and Control

Two tubes were prepared (A= total, B= control), each containing 1 ml each of buffer and substrate. The tubes were placed in a water bath at 37^0C and after about 3 min, 0.1 ml of serum was added to tube A. The content was mixed well and the incubation was allowed to proceed for an hour. Hydrolysis was stopped by the addition of 1ml 0.5 N NaOH to tube A and B and the content was missed thoroughly. Now 0.1 ml of serum was added to control tube B, and 2 ml of A.A.P. reagent followed by 1 ml of ferricyanide was added to each tube. The contents of the tubes were mixed well after each addition.

Standard

This was having the composition: 1.1 ml buffer + 1 ml of dilute standard phenol solution + 1ml 0.5 N NaOH + 2 ml A.A.P. reagent + 1 ml ferricyanide.

Blank

Blank was similar to the standard except water was used in place of the phenol solution.

The color developed immediately and was stable for at least an hour if kept in dim light. The absorbance of the color developed was recorded spectrophotometrically at 510 nm.

Calculation

Serum acid phosphatase activity in KA units per 100 ml was calculated as:

$$\text{Serum acid phosphatase activity} = \frac{\text{O.D. Test} - \text{O.D. Control}}{\text{O.D. Standard} - \text{O.D. Blank}} \times 10$$

3.18.7 Serum lipid peroxidation

Serum lipid peroxidation was measured following the method of Okhawa *et al.* (1979). In this method the released malondialdehyde (MDA) serves as an index of lipid peroxidation**.**

Assay procedure

To 0.2 ml of serum sample, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA) aqueous solution were added. Distilled water (0.6 ml) was added to make up the final volume 4ml. The content was vortexed, kept in water bath at 100^0C for 60 min and cooled in ice bath or in tap water for 5 min. To the content 1ml water and 5ml n- butanol/pyridine mixture (15:1 v/v) were added and the content was shaken vigorously. The content was centrifuged at 4,000 rpm for 10 min at 4^0C and the absorbance of organic upper layer was recorded at 532 nm against a reagent blank.

The concentration of thiobarbituric acid reactive substance was expressed as nmoles MDA ml^{-1} of serum using 1,1,3,3- tetraethoxypropane (TEP) as the standard.

3.18.8 Serum glutamate pyruvate transaminase

Serum glutamate pyruvate transaminase (SGPT) in serum samples was assayed according to Reitman and Frenkel (1957) by using kit (Span Diagnostics Ltd.). The brown color of corresponding hydrazone in alkaline medium was measured spectrophotometrically.

Assay procedure

Reagent 1 (0.25ml) was added in each tube. After incubation at 37^0C for 5 min, 0.05 ml of test serum was added to each tube and the content was mixed well followed by 30 min incubation at 37^0C. After that 0.25 ml of reagent 2 was added, the content was mixed well and the tubes were incubated at room temperature for 20 min. At last 2.5 ml of solution-I

was added, the content was mixed well and the tubes were incubated at room temperature for 10 min. Absorbance was recorded at 505 nm against purified water.

The concentration of serum glutamate pyruvate transaminase was expressed as IUL^{-1} of serum using OD of the test on Y – axis of the standard curve and extrapolates it to the corresponding enzyme activity on X – axis.

3.19 Assessment of humoral immune response

Humoral immune response was assessed by determining the serum IgG antibody titre against bovine collagen type II (CII) using enzyme linked immunosorbent assay (ELISA) following the method of Seki et *al.* (1988) with slight modification.

3.19.1 Antigen preparation for ELISA

Collagen from bovine tracheal cartilage type II (CII) (Sigma, USA) dissolved in phosphate buffer saline (PBS) (20 µg ml^{-1}, pH 7.6) at 4^0C overnight was used as antigen for assessing the humoral response.

3.19.2 Coating of ELISA plates with antigen

The wells of flat bottom microplates were coated each with 50 µl of antigen and the antigen-coated plates were incubated at 4^0C for overnight. These plates were washed thrice with PBS-Tween 20 (pH 7.6), blocked with 1% BSA, PBS-Tween 20 solution and incubated at room temperature for 2 h. After that the plates were again washed thrice with PBS-Tween 20.

3.19.3 Preparation of serum sample dilution and analysis

Five µl of test serum was added to 1ml of dilution buffer to get dilution of 1:200. The diluted serum (100 µl) was added to the wells of ELISA plate; the plate was incubated at room temperature for 2 h and washed thrice with PBS-Tween 20. Soaking time of 3 min was allowed to each wash. The 100 µl of diluted (1:1000) horse radish peroxidase (HRPO) anti-rat IgG conjugate (Sigma, USA) was added to the well and the plate was incubated at room temperature for 1 h. The plate was rewashed thrice with PBS-Tween 20. Substrate solution comprising of citric acid- phosphate buffer (pH 5.0), 20 ml; orthophenylene diamine hydrochloride (OPD), 10 mg; and 30% H_2O_2, 40 µl were added to the wells of ELISA plate. The plates were incubated at room temperature in dark for 20 min. The reaction was stopped by adding 1 M H_2SO_4. The absorbance was recorded at a wavelength of 490 nm using ELISA plate reader (BIO-RAD).

3.20 Assessment of cellular immune response

3.20.1 Delayed type hypersensitivity response

Delayed type hypersensitivity (DTH) response in control, arthritic and *S. platensis* treated arthritic rats was determined according to Banerjee *et al.* (1988). On 35th day after CII immunization, whereas 10 μg of CII in phosphate buffer was injected in one ear, buffer alone was injected in the other ear of normal control, arthritic control and *S. platensis* treated arthritic rats. Measurements of each ear were taken with a micrometer before and after 24 h of injection.

DTH reaction of each rat was measured as:

[(Thickness of ear at 24 h after injecting C II- Thickness of ear before injecting C II) - (Thickness of ear at 24 h after injecting buffer alone - Thickness of ear before injecting buffer alone)]

3.20.2 Pro-inflammatory cytokine level

It includes the determination of pro-inflammatory cytokine (TNF-α, IL- 1β,) in serum sample of normal control, arthritic control, *S. platensis* and methotrexate treated arthritic rats.

3.20.2.1 Rat tumor necrosis factor alpha (TNF-α) ELISA test

The Endogen rat tumor necrosis factor alpha (TNF-α) ELISA kit which is an *in vitro* ELISA for the quantitative measurement of rat TNF-α in serum was used in this assay.

Methodology

Sample dilution

Serum was first diluted to 1:1 before testing. To prepare 1:1 dilution, 100 µl of sample was added to an equal amount of standard diluent in a separate tube and mixed well.

Reagent preparation

Wash buffer

A clean glass or plastic container (2 liter) was used for preparing and storing the wash buffer. The entire content (50 ml) of wash buffer (30X) bottle was transferred to 2 liter container and the final volume was made to 1.5 liter with ultra pure water. The wash buffer solution was mixed thoroughly prior to use.

Standards

When testing serum samples, the standard was reconstituted with ultra pure water to the volume indicated on the standard vial label. Standard was dissolved approximately within 1 min and was mixed by gently inverting the vial. The standard diluent was used to prepare standard curve serial dilutions. Six tubes were labeled, one for each standard curve point as 2,500, 833, 278, 93, 31, and 0 pg ml^{-1}. An initial 1:6 dilution was prepared followed by 1:3 serial dilutions for the standard curve as follows: Appropriate diluent (600 µl) was pipetted into each tube. Reconstituted standard (120 µl) was pipetted into the first tube (i.e. 2,500 pg ml^{-1}) and mixed properly. 300 µl of this dilution was pipetted into the second tube (i.e. 822 pg ml^{-1}) and mixed thoroughly. Serial dilutions (using 300 µl) were done 3 more times to complete the standard curve points.

Assay procedure

Sample incubation

Pre-treatment buffer (50 µl) was added to each well. Reconstituted standard or diluted sample (50 µl) was added to each well in duplicate. The content was mixed well by gently tapping the plate several times. Standard diluent (50 µl) was added to all the wells that did not contain either standard or samples. Plate was carefully covered with an adhesive plate cover and incubated for 1 h at room temperature (i.e. 20-25^{0}C). The adhesive plate cover was carefully removed. Plate was washed 3 times with wash buffer and after that the plate was blotted onto paper towels or other absorbent material.

Biotinylated antibody reagent incubation

Biotinylated antibody reagent (50 µl) was added to each well. A new adhesive plate cover was carefully attached. Plate was incubated for 1 h at room temperature. The adhesive plate cover was carefully removed; plate content was discarded and washed thrice.

Streptavidin-HRP reagent incubation

Streptavidin-HRP reagent (100 µl) was added to each well. A new adhesive plate cover was carefully attached. Plate was incubated for 30 min at room temperature. The adhesive plate cover was carefully removed; plate content was discarded and washed thrice.

Substrate incubation and stop step

TMB substrate solution (100 µl) was pipetted into each well and the color reaction was allowed to develop at room temperature in the dark for 10 min.

Plate was not covered with a plate sealer. The substrate reaction yielded a blue solution that turned yellow when stop solution was added. After 10 min, the reaction was stopped by adding 100 µl of stop solution to each well.

Absorbance measurement

The plate was evaluated within 30 min of stopping the reaction. Absorbance was recorded on ELISA plate reader (BIO-RAD) set at 450 and 550 nm.

The absorbance data recorded at 550 nm was subtracted from the absorbance data recorded at 450 nm to correct for optical imperfections in the microplate.

Sensitivity: <15 pg ml^{-1}

Calculation: The quantity of TNF-α was determined according to the linear regression equation of the standard curve.

3.20.2.2 Rat interleukin-1β ELISA

The Endogen rat interleukin-1 beta (IL-1β) ELISA kit which is an *in vitro* ELISA for the quantitative measurement of rat IL-1β in serum samples was used in this assay.

Methodology

Sample dilution

Serum was first diluted to 1:1 before testing. To prepare 1:1 dilution, 100 µl of sample was added to an equal volume of standard diluent in a separate tube and mixed well.

Reagent preparation

Wash buffer

A clean glass or plastic container (2 litre) was used for preparing and storing the wash buffer. The entire content (50 ml) of wash buffer (30X) bottle was transferred to 2 litre container and the final volume was maintained to 1.5 litre with ultra pure water. The wash buffer solution was mixed thoroughly.

Standards

Standards were prepared just before use and used within 1 h of reconstitution. Standard was reconstituted with ultra pure water to the volume indicated on the standard vial label. Standard was mixed by gently inverting the vial until the contents were completely dissolved. The sample diluent was used to prepare standard curve serial dilutions. Seven tubes were labeled, one for each standard curve point as 2,500, 1,000, 160, 64, 25.6, and 0 pg ml^{-1}. A 1:2 dilution was prepared to generate the 2,500 pg ml^{-1} standard. After that 1:2.5

serial dilution was also prepared for additional standard curve points as follows: 240 µl of sample diluent was pipetted into each tube. After that 240 µl of the reconstituted standard was pipetted into the first tube (i.e. 2,500 pg ml^{-1}) and the content was mixed. 160 µl of this dilution was pipetted into the second tube (i.e. 1,000 pg ml^{-1}) and the content was again mixed thoroughly. Serial dilutions (using 160 µl) were repeated four more times to complete the standard curve points. 2,500, 1,000, 400, 160, 64, 25.6 and 0 pg ml^{-1} concentrations were used as the standard curve points.

Assay procedure

Sample incubation

Sample diluent (50 µl) was added to each well. Reconstituted standards or test samples (50 µl) were added in duplicate to each well. The contents were mixed well by gently tapping the plate several times. Plate covered carefully with a new adhesive plate cover was incubated for 2 h at room temperature (20-25^{0}C). Adhesive plate cover was carefully removed, plate content was discarded and the plate was washed five times with wash buffer.

Biotinylated antibody reagent incubation

Biotinylated antibody reagent (100 µl) was added to each well containing sample or standard. A new adhesive plate cover was carefully attached and the plate was incubated for 30 min at room temperature (20-25^{0}C). Adhesive plate cover was carefully removed, plate content was discarded and plate was washed five times with wash buffer.

Streptavidin-HRP solution preparation and incubation

Streptavidin-HRP solution was prepared immediately before use. Only the streptavidin-HRP solution amount required was used for the number of strips being used. For each strip, 2.5 µl of concentrated steptavidin-HRP was mixed with 1 ml of streptavidin-HRP dilution buffer. Prepared streptavidin-HRP solution (100 µl) was added to each well. A new adhesive plate cover was carefully attached and the plate was incubated for 30 min at room temperature (20-25^{0}C). The adhesive plate cover was carefully removed, plate content was discarded and the plate was washed five times.

Substrate incubation and stop step

TMB substrate solution (100 µl) was pipetted into each well. Enzymatic color reaction was allowed to develop at room temperature in the dark for 30 min. Plate was not covered with aluminum foil or a plate sealer. The substrate reaction yielded a blue solution that turns

yellow when stop solution was added. After 30 min, the reaction was stopped by adding 100 µl of stop solution to each well.

Absorbance measurement

The plate was evaluated within 30 min of stopping the reaction. Absorbance was recorded on an ELISA plate reader set at 450 and 550 nm. The absorbance data recorded at 550 nm was subtracted from the absorbance data recorded at 450 nm to correct for optical imperfections in the microplate.

Sensitivity: <12 pg ml^{-1}

Calculation: The quantity of IL-1β was determined according to the linear regression equation of the standard curve.

3.21 Statistical analysis

The values were presented as mean±SEM of six rats per group. Results were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey and Holm-Sidak test, all pair wise multiple comparison procedure sigma stat 3.5, software Inc. (USA). A value of *****$P<0.05$ was considered significant to arthritic control vs. treated groups. $^{\#}P<0.05$ was considered significant to normal control vs. arthritic control.

Results

4.1 Biomass preparation of *S. platensis*

S. platensis was routinely grown in Zarrouk's medium (Zarrouk, 1966) in culture flasks. The cultures were incubated and maintained in a culture room at 25^0C and illuminated with day-light fluorescent tubes having the photon fluence rate of 50 μmol m^{-2} s^{-1} on the surface of the vessels, for obtaining the optimum growth. *S. platensis* biomass was filtered (after 3 weeks, the period showing maximum growth), dried and stored in sealed pack container. The dried biomass was grinded with pestle and mortar into powdered form and used as oral feed for experimental rats post mixing with distilled water for the purpose of therapeutic treatment for experimental rats.

4.2 Behavior examination

4.2.1 Arthritis assessment

The sign of arthritis was started from 15^{th} day post collagen immunization with slight swelling and redness, reached at significant level at 20^{th} day and attained its maximum level at 45^{th} day. The macroscopic sign of severe arthritis at 45^{th} day included swelling, redness, deformity and ankylosis in hind paws and ankle joints. Such symptoms were, however, found in fore limbs as well. The hind paw of normal rats showed significant difference with the hind paw of arthritic control rats. Whereas, arthritic rats treated with *S. platensis* (800 mg kg^{-1}) showed redness and swelling only, the arthritic rats treated with *S. platensis* (400 mg kg^{-1}), however, showed almost no sign of arthritis and appeared essentially similar to normal rats. In contrast, methotrexate (0.3 mg kg^{-1}) treated arthritic rats showed redness and swelling with moderate arthritis at the 45^{th} day when the animals were sacrificed (Fig.1).

4.2.2 Arthritic score

A significant increment in arthritic score was observed in arthritic control rats from 15^{th} (1.75±0.31), 20^{th} (3.5±0.22), 30^{th} (3.96±0.05), 40^{th} (3.92±0.08) and 45^{th} day (3.92±0.08) during development of arthritis. However, *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in arthritic score from 25^{th} (3.5±0.22), 30^{th} (2.33± 0.32), 35^{th} (1.67±0.20), 40^{th} (1.0±0.28) and 45^{th} day (0.50±0.22) during development of arthritis as compared to the arthritic score of their arthritic control counterparts. In contrast, *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats showed lower arthritic score as compared to the arthritic score of their arthritic control

counterparts but they did not show significant decline at P≤0.05 level during development of arthritis (Fig.2).

4.2.3 Change in Paw thickness

A significant increment in hind paw thickness from 15th (5.53±0.47 mm), 20th (6.22±0.29 mm), 30th, 40th, and 45th day (5.9±0.21 mm) was observed in arthritic control rats during development of arthritis. Arthritic control rats, however, showed a significant difference in hind paw thickness from normal control rats from 3.47±0.13 to 4.05±0.06 mm on 15th to 45th day, respectively. Whereas, *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in paw thickness from 30th (4.55±0.41 mm) to 45th day (4.2±0.22 mm), the arthritic rats treated with *S. platensis* (800 mg kg^{-1}), however, showed significant decline in paw thickness from 35th (4.65±0.29 mm) to 45th day (4.48±0.25 mm) as compared to the paw thickness of their arthritic control counterparts during development of arthritis. The marketed drug methotrexate (0.3 mg kg^{-1}) treated arthritic rats also showed significant decline in paw thickness from 40th (5.02±0.21 mm) to 45th day (4.95±0.23 mm) as compared to the paw thickness of their arthritic control counterparts during development of arthritis (Fig.2).

4.2.4 Ankle size

A significant increment in ankle size of arthritic control rats was recorded from 20th (0.6±0.01 cm) to 45th day (0.68±0.01 cm) as compared to the ankle size of their normal control counterpart [20th (0.36±0.004 cm) to 45th day (0.5±0.008 cm)].

Whereas, *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in their ankle size (0.51±0.006 cm) at 45th day, the arthritic rats treated with *S. platensis* (800 mg kg^{-1}), however, showed non-significant decline in their ankle size from 25th (0.64 ±0.04 cm) to 45th day (0.57±0.03 cm) as compared to the ankle size of their arthritic control counterparts. Methotrexate (0.3 mg kg^{-1}) treated arthritic rats also showed decline in their ankle size from 25th (0.74±0.04 cm) to 45th day (0.63±0.02 cm) as compared to the ankle size of their arthritic control counterparts, but these decrease in ankle size was, however, found to be statistically non-significant at P≤0.05 (Fig. 3).

4.2.5 Change in body weight

The data in Fig.3 show changes in body weight of normal control, arthritic control, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats before (0 day) and after collagen immunization. An absolute increment in the body weight of all the groups of rats was found to be almost similar in the first 12 days and no significant differences were observed between them. However, after 12 days, a loss in the body weight was observed in the arthritic control, *S. platensis* and methotrexate treated arthritic rats from 15^{th} to 20^{th} day as compared to the body weight of their normal control counterparts. The body weight of arthritic control rats declined significantly from 15^{th} (143.33±1.63 g) to 45^{th} day (147±1.66 g) as compared to the body weight of their normal control counterpart. *S. platensis* (400 mg kg^{-1}) treated arthritic rats showed significant increment in their body weight as compared to their arthritic control counterparts from 30^{th} (158.67±4.30 g) to 45^{th} day (171.33±0.60 g). Arthritic rats treated with *S. platensis* (800 mg kg^{-1}) whereas, showed significant increment in their body weight from 30^{th} (157.67±4.51 g) to 45^{th} day (169.0±0.89 g), methotrexate (0.3 mg kg^{-1}) treated arthritic rats, however, showed significant increment in their body weight on 25^{th} (153.33±2.60 g), 30^{th} (156.33± 3.11 g), 40^{th} (160.0±2.07 g) and 45^{th} day (162.0±1.03 g) as compared to the body weight of their arthritic control counterparts.

4.2.6 Arthritic pain

The arthritic pain was measured in normal control, arthritic control, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats during development of arthritis. A significant increment in arthritic pain was observed in arthritic control rats from 20^{th} (2.75±0.29) to 45^{th} day (3.83±0.01) during development of arthritis as compared to the arthritic pain of their normal control counterparts. However, Oral dose of *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in arthritic pain from 40^{th} (1.25±0.16) to 45^{th} day (0.50±0.22) during development of arthritis. In contrast, *S. platensis* (800 mg kg^{-1}) and marketed drug methotrexate (0.3 mg kg^{-1}) treatment to the arthritic rats resulted in a decline in the arthritic pain but did not show significant difference in their arthritic pain of their arthritic control counterparts at $P \leq 0.05$ level during development of arthritis (Fig.4).

4.2.7 1-5 toe spread

Functional assessments of rats were performed using walking track analysis. Individual walking toe spread (1-5 toe spread) values (in cm) were measured in hind paws of normal control, arthritic control, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats at 45^{th} day post collagen immunization. A statistical significant difference in 1-5 toe spread was recorded in arthritic control rats (0.94±0.067) as compared to their normal control (1.52±0.057) counterparts at the end of the experiment (45^{th} day). A significant spread between 1-5 toe was observed in the arthritic rats treated with *S. platensis* 400 mg kg^{-1} (1.51±0.04) and 800 mg kg^{-1} (1.41±0.04) respectively, as compared to their arthritic control counterparts (0.94±0.067) at 45^{th} day post collagen immunization. Whereas, no statistical significant difference in 1-5 toe spread was, however, observed in marked drug methotrexate (0.3 mg kg^{-1}) treated arthritic rats (1.05±1.10) as compared to the 1-5 toe spread of their arthritic control counterparts (Fig. 4).

4.2.8 2-4 toe spread

The measurement of functional recovery in 2-4 toe spread was recorded using foot prints of hind paws.The changes in 2-4 toe spread (in cm) of hind paws of normal control, arthritic control, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats at 45^{th} day post collagen immunization. A significant reduction in 2-4 toe spread was observed in arthritic control rats as compared to the 2-4 toe spread of their normal control counterparts. A significant spread between 2-4 toe was observed in the arthritic rats treated with 400 mg kg^{-1} (0.69±0.02) and 800 mg kg^{-1} (0.66±0.07) *S. platensis,* respectively, at 45^{th} day post collagen immunization. No significant difference in 2-4 toe spread was, however, observed in methotrexate (0.3 mg kg^{-1}) treated arthritic rats (0.53±0.04) as compared to 2-4 toe spread of their arthritic control counterparts (Fig.5).

4.2.9 Total print length

Functional assessments of rats were performed using walking track analysis. Individual walking total print length values (in cm) were measured in normal control, arthritic control, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats at 45^{th} day post collagen immunization. Oral dose of *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats affected all footprint

measurements. A significant reduction in total print length was observed in arthritic control rats (1.78±0.08) at 45th day post collagen immunization as compared to their normal control (2.72±0.05) counterparts. A significant difference in total print length was recorded in 400 (2.82±0.22) and 800 mg kg^{-1} (2.55±0.06) *S. platensis* treated arthritic rats at 45th day. No significant difference in total print length was, however, observed in methotrexate (0.3 mg kg^{-1}) treated arthritic rats (2.41±0.13) as compared to the total print length of their arthritic control counterparts. Thus, individual total print length measurements alone can be used to characterize functional recovery post inflammation (Fig.5).

4.3 Radiological analysis

The hind paws of rats were used for radiological scoring. All radiographs were taken with x-ray film (Kodak Diagnostic Film). Radiographic severity of joint destruction was examined at the end of the experiment (45th day). Bone erosion and joint space narrowing were detected in the ankle joint of arthritic rats. Arthritic changes were significantly reduced in *S. platensis* (400 mg kg^{-1}) treated arthritic rats when compared with their arthritic control rats. Whereas, *S. platensis* (800 mg kg^{-1}) treatments to the arthritic rats resulted in decrease in the soft tissue swelling score, methotrexate treatment to the arthritic rats, however, showed decreased inflammation but the inflammation level remained slightly higher than those with *S. platensis* (400 mg kg^{-1}) treated rats (Fig.6).

4.3.1 Radiological score

Radiographic arthritic changes in hind paws of experimental rats were measured on the basis of tissue swelling, oedema, joint erosion, bone erosion and osteophyte formation at 45th day post collagen immunization. A statistically significant difference in radiological score was recorded in arthritic rats (2.0±0.33) as compared to their normal control counterparts. *S. platensis* (400 mg kg^{-1}) treatments to arthritic rats resulted in a significant reduction in the arthritic symptoms (0.17±0.20) when compared with their arthritic control counterparts. *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats whereas decreased the radiological score (0.50±0.22) and (0.83±0.28), respectively, no significant difference was, however, recorded at $P \leq 0.05$ level as compared to arthritic control counterparts (Fig. 7).

4.4 Histopathological study

Histology of the joints of arthritic control rats showed vigorous proliferation of synovial cells, resulting in pannus formation and infiltration of mononuclear cells and neutrophils to the subsynovial region. Pannus destroyed the cartilage and bone. In contrast, normal rats showed normal histology without any synovial infiltration, pannus formation, and cartilage and bone destruction. *S. platensis* (800 mg kg^{-1}) treated arthritic rats showed minimal to moderate synovial cell infiltration with less destruction of cartilage, however, they did not show any bone destruction. *S. platensis* (400 mg kg^{-1}) treated arthritic rats showed almost no sign of synovial cell infiltration, pannus formation, synovitis, and cartilage and bone destruction. Methotrexate (0.3 mg kg^{-1}) treated arthritic rats, however, showed moderate synovial cell infiltration with less destruction of cartilage and slight bone destruction. Microscopic analysis of phalangial joint articulations showed that the right and left paws of normal rats were clear of any signs of inflammation. In contrast, the joint of arthritic control rats had moderate to severe synovitis, considerable inflammatory cell infiltration into mineralized and non-mineralized tissues. Bone resorption in these rats was moderate, with minimal periosteal new bone formation. In arthritic rats treated with *S. platensis* (400 mg kg^{-1}), synovitis and inflammatory cell influx was minimal with no sign of cartilage ulceration. *S. platensis* (800 mg kg^{-1}) treated arthritic rats had moderate synovitis, minimal to moderate inflammatory cell infiltration into tissues, and no signs of cartilage ulceration. Bone resorption was moderate with a lower degree of periosteal new bone formation. Methotrexate (0.3 mg kg^{-1}) treated arthritic rats showed moderate synovitis, moderate cell inflammation into tissue with less destruction of cartilage and few signs of cartilage ulceration. Bone resorption was, however, found to be moderate (Fig.8).

4.5 Biochemical analysis

4.5.1 Total serum cholesterol

The data show the total serum cholesterol levels in normal control, arthritic control and *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats at 45^{th} day post collagen immunization. It is evident from the data of Fig 16B that total serum cholesterol level in arthritic control rats was found to be significantly higher (75.83±2.92 $mgdl^{-1}$) as compared to the total serum cholesterol level (39.04±3.54 mg dl^{-1}) of their normal control counterparts at 45^{th} day. *S. platensis* (400 and 800mg kg^{-1}) and methotrexate (0.3 mg

kg^{-1}) treatments to the arthritic rats resulted in a significant decline in their serum cholesterol levels of 50.89 ±2.48, 54.21 ±1.31 and 57.84±6.77 mg dl^{-1}, respectively at 45^{th} day as compared to the serum cholesterol levels of their arthritic control counterparts (Fig.9).

4.5.2 Total serum albumin

A significant decline in the total serum albumin level (3.12±0.07 gdl^{-1}) was recorded in arthritic control rats as compared to the total serum albumin level (4.96±0.55 g dl^{-1}) of their normal control counterparts at 45^{th} day. Methotrexate (0.3 mg kg^{-1}) treated arthritic rats, however, did not show any significant difference in their serum albumin level (4.00±0.28 g dl^{-1}) at 45^{th} day as compared to the serum albumin level of their arthritic control counterparts. However, arthritic rats treated with *S. platensis* (400 and 800 mg kg^{-1}) showed significant increase in serum albumin level (4.93±0.47 and 4.82±0.43 g dl^{-1}) at 45^{th} day as compared to the serum albumin level of their arthritic control counterparts (Fig.9).

4.5.3 Total plasma protein

The levels of total plasma protein in arthritic control (7.50±0.12 g dl^{-1}) and normal (7.10±0.15 g dl^{-1}) rats did not show any significant difference at 45^{th} day post collagen immunization. Whereas, arthritic rats treated with 800 and 400 mg kg^{-1} *S. platensis* showed total plasma protein levels of 7.52±0.09, 7.50±0.14 g dl^{-1}, respectively, methotrexate (0.3 mg kg^{-1}) treated arthritic rats, however, showed reduction in their plasma protein level (7.32±0.16) at 45^{th} day which was found to be non significant (Fig. 10).

4.5.4 Total serum protein

Total protein level in the serum samples of arthritic control rats was found to be 6.63±0.29 g dl^{-1} as compared to the total serum protein level of 6.08±0.45 g dl^{-1} of normal rats at 45^{th} day post collagen immunization. However, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats showed increase in their serum protein levels of 6.98±0.34, 7.0±0.45 and 6.66±0.24, respectively as compared to the serum protein levels of their arthritic control counterparts. No statistically significant difference in total serum protein levels was, however, recorded in these groups (Fig.10).

4.5.5 Serum alkaline phosphatase activity

A significant increment in the serum alkaline phosphatase activity (62.47±7.33 KA unit) was recorded in arthritic control rats at 45^{th} day post collagen immunization with respect to

the serum alkaline phosphatase activity of their normal control counterparts (32.46±4.70 KA unit). However, *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline of their serum alkaline phosphatase activity (28.02±1.59 KA unit) at 45^{th} day as compared to the serum alkaline phosphatase activity of their arthritic control counterparts. Arthritic rats treated with *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}), however, did not show significant decline in their serum alkaline phosphatase activity of 36.01±4.12 and 38.88±8.53 KA unit, respectively at 45^{th} day as compared to the serum alkaline phosphatase activity of their arthritic control counterparts (Fig.11).

4.5.6 Serum acid phosphatase assay

A significant increment in the acid phosphatase activity (6.90±0.30 KA unit) was recorded in arthritic control rats at 45^{th} day with respect to their normal control counterparts having acid phosphatase activity of 4.26±0.19 KA unit at 45^{th} day. *S. platensis* (400 mg kg^{-1}) therapeutic treatments to the arthritic rats resulted in a significant decline in their serum acid phosphatase activity (5.08±0.19 KA unit) at 45^{th} day as compared to the serum acid phosphatase activity of their arthritic control counterparts. *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats also resulted in a significant decline in their serum acid phosphatase activity of 5.46±0.39 and 5.39±0.30 KA unit, respectively as compared to the serum acid phosphatase activity of their arthritic control counterparts at 45^{th} day (Fig. 11).

4.5.7 Serum lipid peroxidation

A highly significant elevation in the serum lipid peroxidation (LPO) level (5.62±0.29 MDA nmoles ml^{-1}) was recorded in arthritic control rats at 45^{th} day with respect to their normal control counterparts having LPO level of 2.87±0.21 at 45^{th} day. *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in their serum LPO level (3.59±0.26 MDA nmoles ml^{-1}) at 45^{th} day as compared to the serum LPO levels of their arthritic control counterparts. In contrast, arthritic rats treated with *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}), however, did not show any significant decline in their serum LPO levels of 4.78±0.30 and 4.83±0.33 MDA nmoles ml^{-1}, respectively as compared to the serum LPO levels of their arthritic control counterparts at 45^{th} day (Fig. 12).

4.5.8 Serum glutamate pyruvate transaminase

A highly significant elevation in serum glutamate pyruvate transaminase (SGPT) concentration (0.16±0.003 IUL^{-1}) was recorded in arthritic control rats at the end of the experiment (45^{th} day) as compared to the SGPT level (0.104±0.002 IUL^{-1}) of their arthritic control counterparts (Fig 20). *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in SGPT concentration (0.105±0.003 IUL^{-1}) at 45^{th} day as compared to the SGPT concentration of their arthritic control counterparts. *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats also resulted in significant decline in their serum SGPT concentration of 0.108±0.001 and 0.137±0.004 IUL^{-1}, respectively as compared to the SGPT concentration of their arthritic control counterparts at 45^{th} day (Fig. 12).

4.6 Analysis of immune responses

4.6.1 Anti- collagen IgG antibody level

A significant increase in the serum anti-collagen IgG antibody level (4.81±0.31) was recorded in arthritic control rats with respect to the serum anti-collagen IgG level (0.29±0.02) of their normal control counterparts at 45^{th} day post collagen immunization. *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats whereas, resulted in a slight decline in the serum anti-collagen IgG antibody levels as compared to the serum anti-collagen IgG antibody level of their arthritic control counterparts at 45^{th} day, the decline was, however, found to be statistically non significant (Fig.13).

4.6.2 Delayed type hypersensitivity response

The mean delayed type hypersensitivity (DTH) response (0.75±0.05 mm) of arthritic control rats was found to be significantly higher than the DTH response (0.03±0.02 mm) of their normal control counterparts. *S. platensis* (800 and 400 mg kg^{-1}) treatments to the arthritic rats resulted in a decline in the mean DTH response of 0.68±0.04 and 0.63±0.05 mm, respectively, as compared to the DTH response (0.75±0.05 mm) of their arthritic control counterparts, the difference in DTH response was, however, found to be statistically non significant. Methotrexate (0.3 mg kg^{-1}) treatment to the arthritic rats also resulted in a non significant decline in their DTH response at 45^{th} day post collagen immunization (Fig. 13).

4.6.3 Rat tumor necrosis factor alpha (TNF-α)

The TNF-α levels were measured in normal control, arthritic control, *S. platensis* (400 and 800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treated arthritic rats at 45^{th} day post collagen immunization. A highly significant elevation in the serum TNF-α concentration (162.00±12.09 pg ml^{-1}) was recorded in arthritic control rats at the end of the experiment (45^{th} day) to that of the TNF-α concentration (31.25±1.05 pg ml^{-1}) of their normal control counterparts at 45^{th} day. *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats resulted in a significant decline in their serum TNF-α concentration (44.0±1.22 pg ml^{-1}) as compared to the serum TNF-α concentration of their arthritic control counterparts. *S. platensis* (800 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats, however, did not show any significant decline in their serum TNF-α concentrations of 70.80±12.22 and 53.50±1.87 pg ml^{-1}, respectively, to that of the serum TNF-α concentration of their arthritic control counterparts at 45^{th} day post collagen immunization (Fig. 14).

4.6.4 Rat interleukin-1β

A highly significant elevation in the serum IL-1β concentration (52.11±5.46 pg ml^{-1}) was recorded in arthritic control rats as compared to the serum IL-1β concentration (29.79±2.68 pg ml^{-1}) of their normal control counterparts at 45^{th} day. *S. platensis* (400 mg kg^{-1}) treatments to the arthritic rats, however, resulted in a significant decrease in their serum IL-1β concentration (33.20±1.93 pg ml^{-1}) at 45^{th} day as compared to the serum IL-1β concentration of their arthritic control counterparts. Whereas, *S. platensis* (800 mg kg^{-1}) treatments to the arthritic rats did not show significant decline in their serum IL-1β concentration (39.51±1.87 pg ml^{-1}) at 45^{th} day, the marketed drug methotrexate (0.3 mg kg^{-1}) treatments to the arthritic rats, however, resulted in a significant decrease in their serum IL-1β concentration (37.89±4.68 pg ml^{-1}) as compared to the serum IL-1β concentration of their arthritic control counterparts at 45^{th} day post collagen immunization (Fig. 14).

Summary

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic disruptive polyarthritis manifestation of chronic inflammation in multiple articular joints, including the knees and small joints of the hands and feet. RA is the commonest inflammatory arthropathy worldwide and affects up to 1 % of the World population. There is no cure for RA and today's treatments aim at achieving the lowest possible level of arthritis disease activity. The most prominent reason for loss of joint mobility and function is chronic or episodic pain, which leads to physiological distress and impaired quality of life. The current systemic anti-TNF-α therapies ameliorate disease in 60 to 70% of RA patients. However, biologics must be given systemically in relatively high dosages to achieve constant therapeutic levels in the joints, and significant side effects have been reported. Biomarkers have been used as surrogate treatment end points in preliminary, short-term, proof-of concept studies, but only limited data concerning biological biomarkers in psoriasis and psoriatic arthritis are available. It has been seen that histological findings are not correlated with clinical disease parameters. A number of additional, non-pharmacologic treatments like therapeutic fasting, dietary supplementation of essential fatty acids and exercise for RA have also been tried. Evidences regarding herbal medications, acupuncture and splinting are inconclusive. Surgery should be considered when pain is unacceptable, loss of motion is significant, or functional impairment is severe.

Recent clinical application of biologic agents targeted to inflammatory cytokines including tumor necrosis factor α (TNF-α) or interleukin-1β (IL-1β) dramatically changed the treatment strategy for RA. These molecular therapies of RA are more effective than the conventional disease-modifying anti-rheumatic drugs (DMARDs) and can even stop the destructive process in some RA patients. Today, low dose of methotrexate is the most commonly used treatment for rheumatic diseases. Weekly low doses of methotrexate act primarily as an anti-inflammatory drug, specifically through the release of adenosine rather than as an anti-metabolite drug as in cancer. An increase of liver enzymes is the most common adverse event observed in clinical practice in arthritis patients under long term use of methotrexate. However, the exact mechanism of such disorder remains unclear. Thus, the

etiology of RA inflammation still remains unknown and there is a demand for developing new therapies with alternative targets.

Spirulina platensis is a filamentous blue-green alga or cyanobacterium that grows particularly in alkaline, brackish and saline waters. *Spirulina* is gaining more attention from medical scientists as a nutraceutical and source of potential pharmaceuticals. *Spirulina* increases production of antibodies, cytokines and other cells that improve immunity and help to ward off infection and chronic illnesses such as cancer and RA. Gamma linolenic acid (GLA) in *Spirulina* is anti-inflammatory and antiproliferative. It is potentially useful for individuals with RA and diabetic neuropathy.

The objective of the present study was to evaluate the therapeutic efficacy of *S. platensis* in collagen induced arthritis (CIA) in rats. Mass cultivation of *S. platensis* was done under continuous illumination in 1 or 2 liter flasks on culture racks with a shaking of 4 to 5 times daily. After filtration, the dried powdered biomass of *S. platensis* was used as oral dietary supplement for experimental rats after mixing with distilled water for the purpose of therapeutic treatment of experimental rats.

On the basis of results of the present study it can be concluded that dietary *S. platensis* is able to suppress the physiological, behavioral, radiological, histological, biochemical and immunological changes produced during CIA in rats. This suppressing ability of dietary *S. platensis* could be due to combined effect of its antioxidant constituents such as phycocyanin, carotenoids, vitamin B_1, $B_{2,}$ C and E and other micronutrients. Therefore, it can be anticipated that *Spirulina* therapy will become a more significant part of the pipeline for developing new therapeutics for rheumatic patients in the future.

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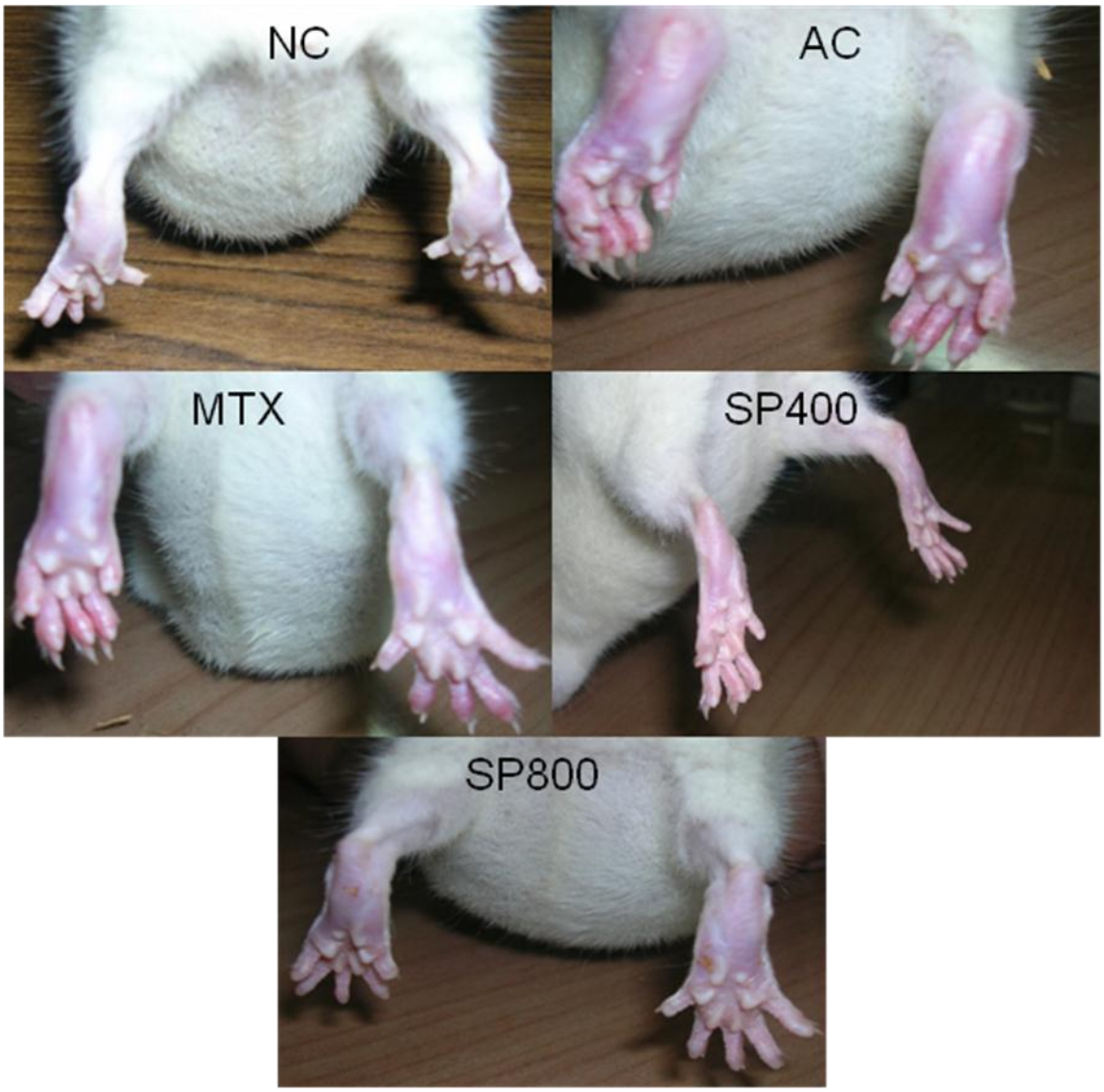
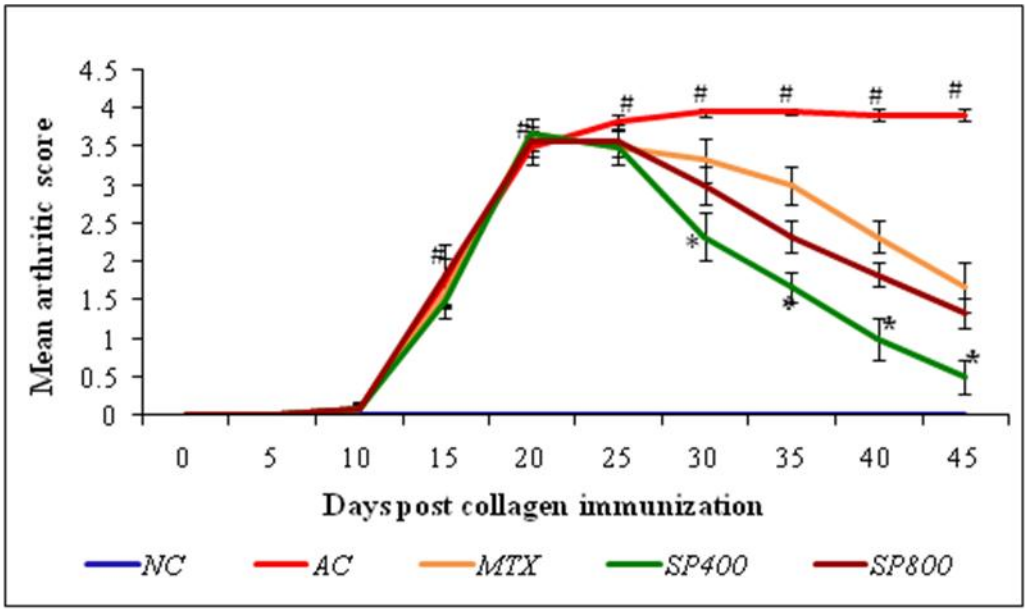


Figure 1. Hind paws and ankle joint of representative rat groups at 45th days. (NC) Normal rat, (AC) Arthritic control rat showing redness, swelling, deformity and ankylosis with severe arthritis (symptoms maximum in the group), (MTX) Methotrexate treated ret with moderate arthritic symptoms , swelling only with moderate arthritis (symptoms maximum in the group), (SP400) *S. platensis* treated (400 mg kg-1) rat showing almost no sign of arthritis and appeared essentially normal (symptoms minimum in the group) and (SP800) *S. platensis* treated (800 mg kg-1) rat showing redness Pictures are representative of six distinct rats per group.



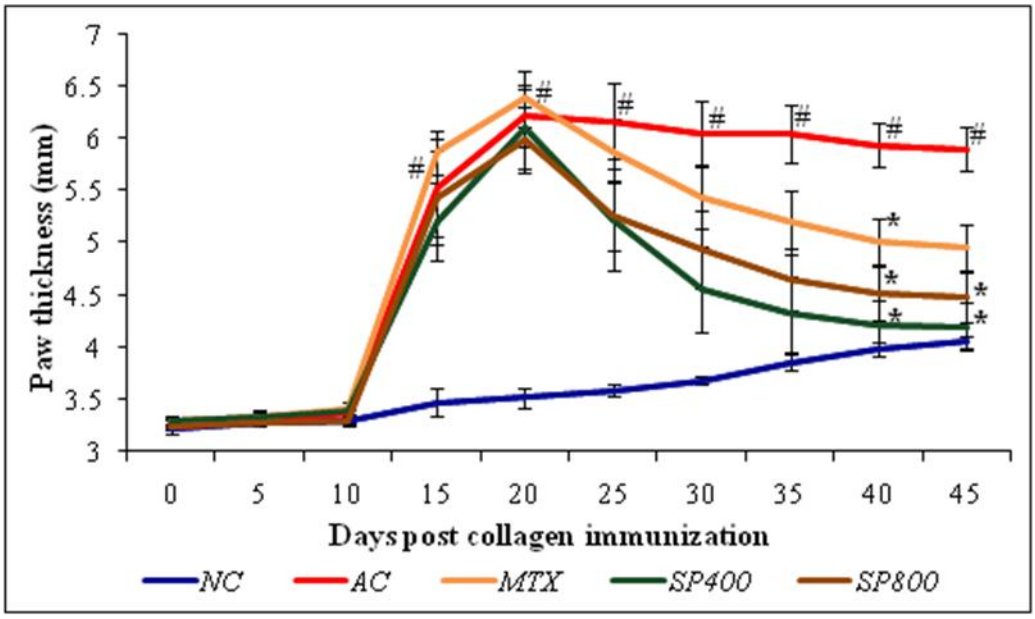
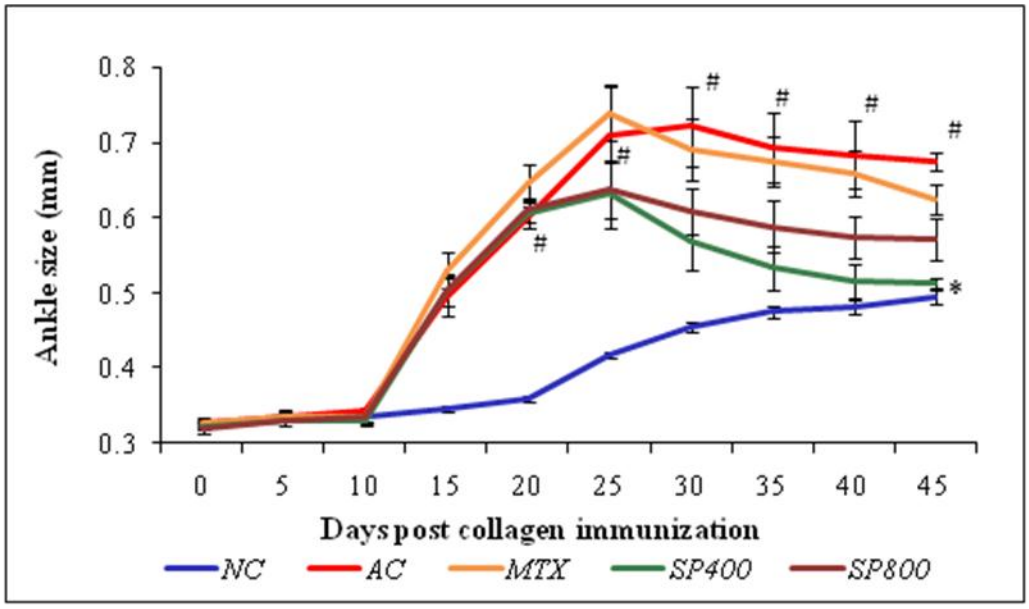


Figure: 2 represent mean arthritic score and paw thickness (nm) from 0 to 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA. *P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



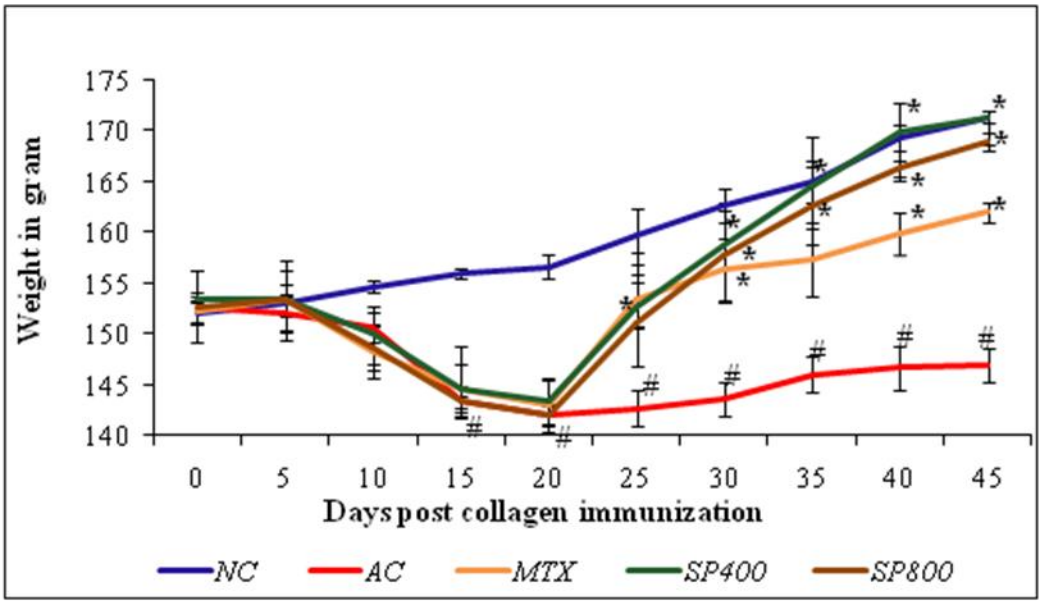
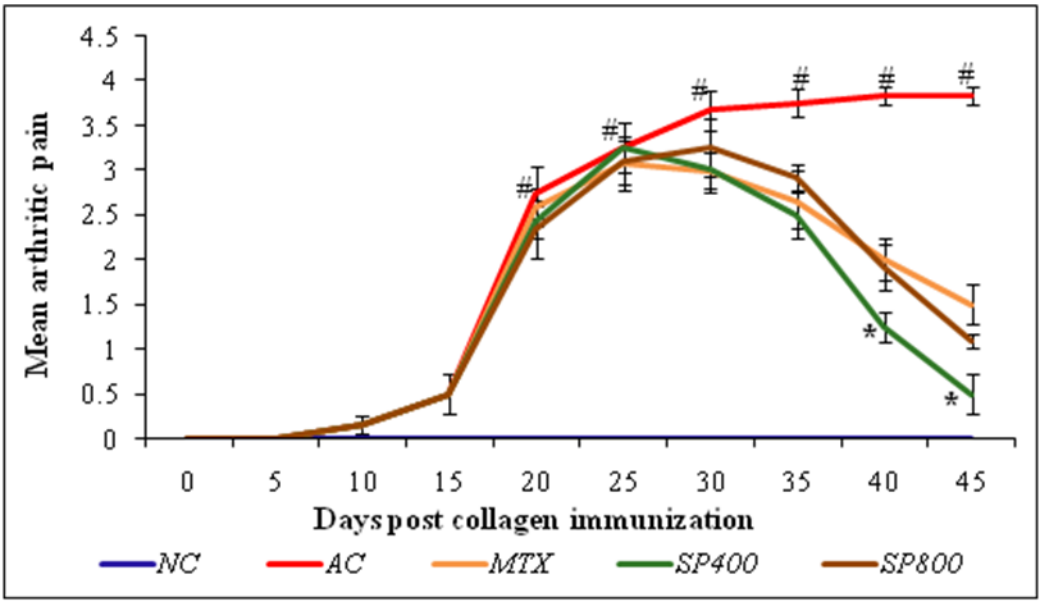


Figure: 3 represent ankle size (mm) and body weight (g) from 0 to 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA. $^{*}P \leq 0.05$ vs AC, $^{\#}P \leq 0.05$ vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



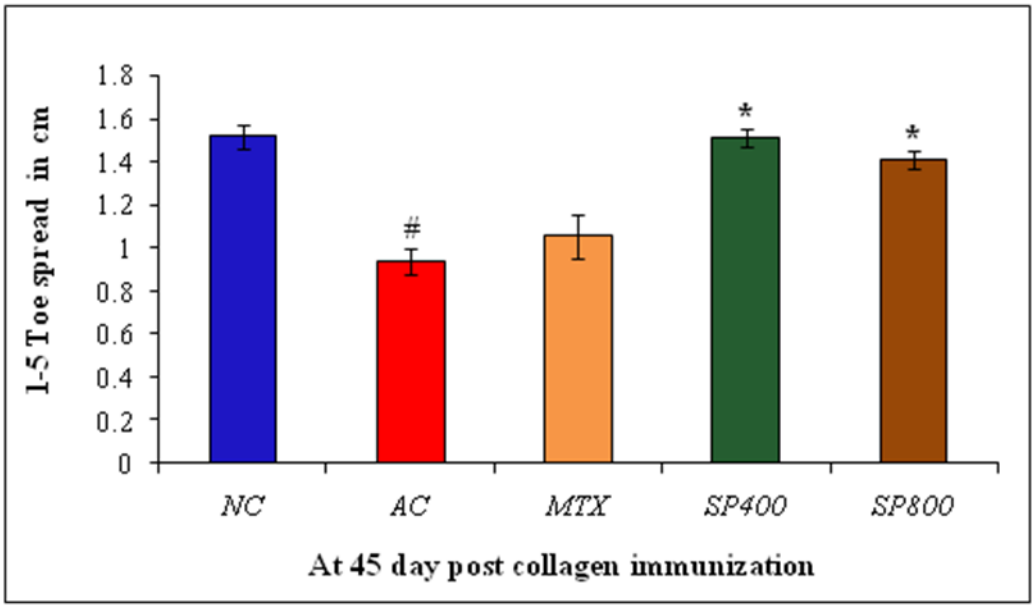
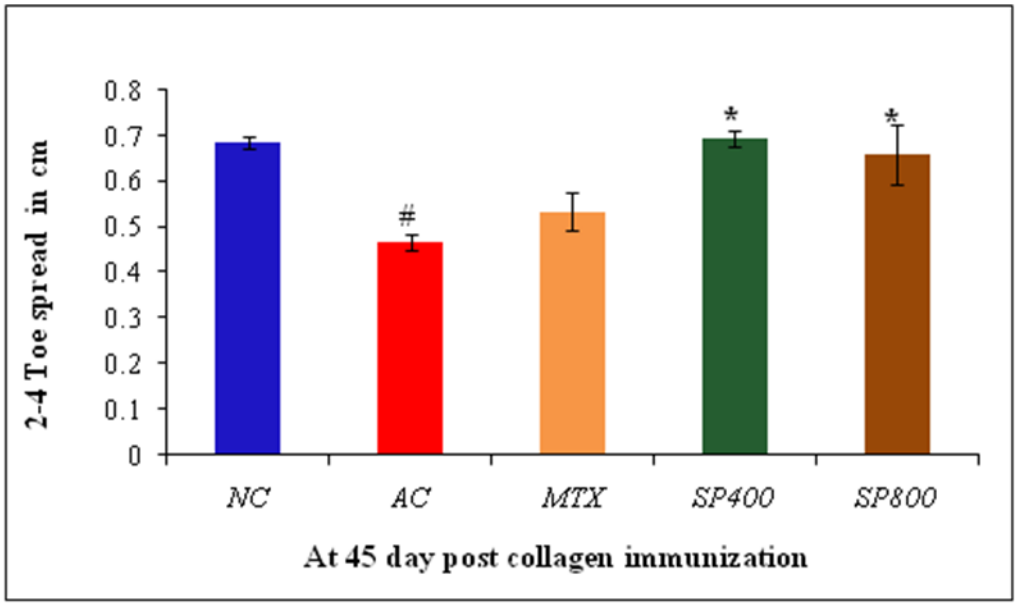


Figure: 4 represent mean arthritic score from 0 to 45 day, and 1-5 toe spread (cm) at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA.
*P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC
NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



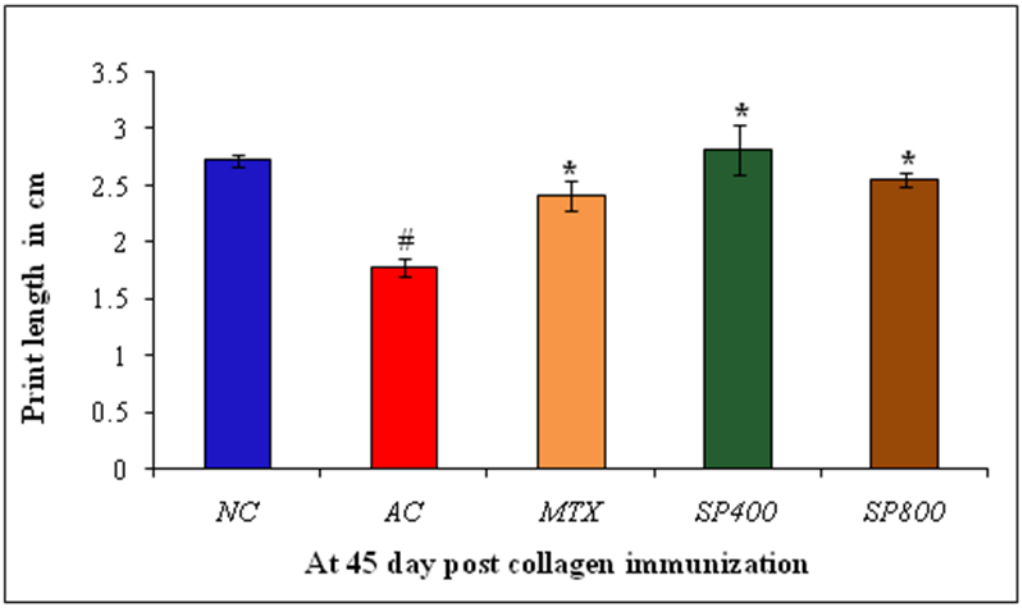


Figure: 5 represent 2-4 toe spread and total print length (cm) from 0 to 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA. $^{*}P \leq 0.05$ vs AC, $^{\#}P \leq 0.05$ vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.

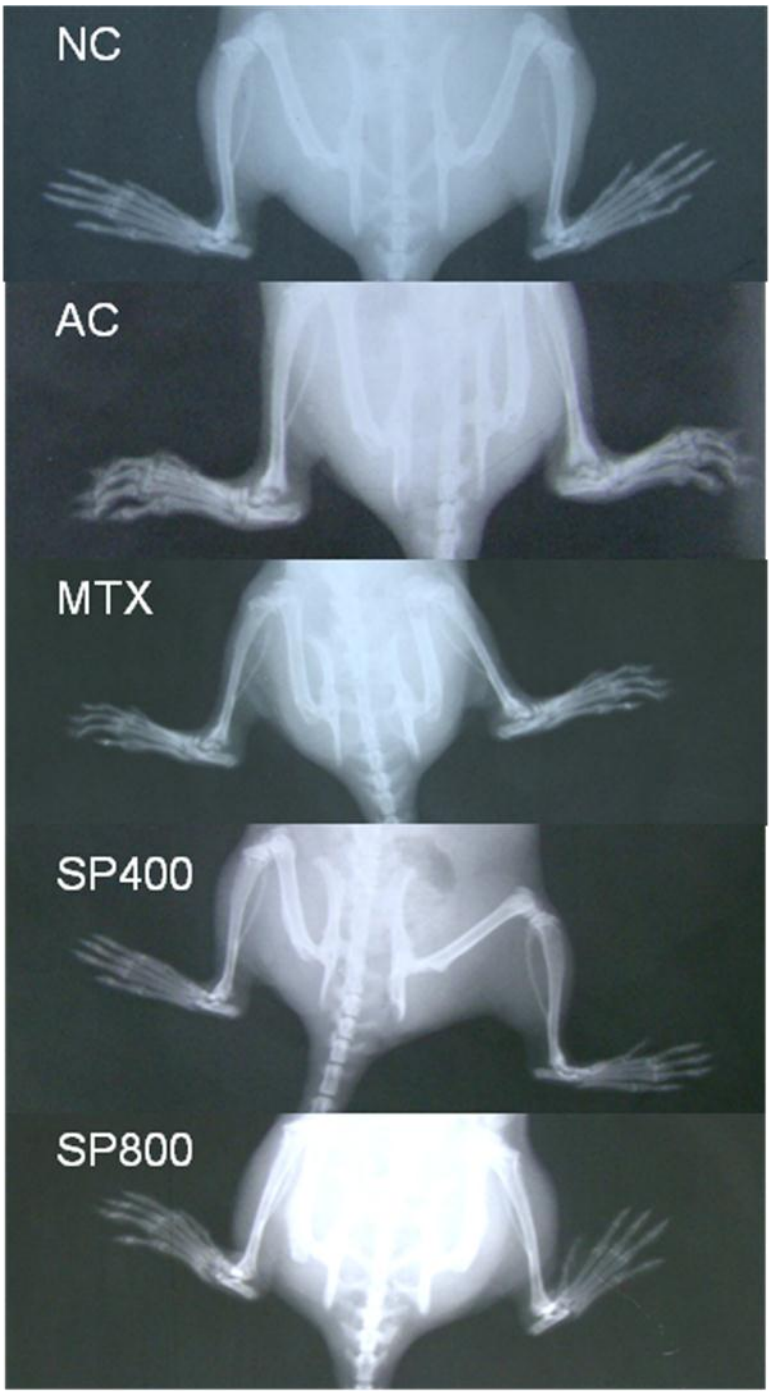


Figure: 6 showed the radiological analysis of joints. X-ray of rats.
NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.

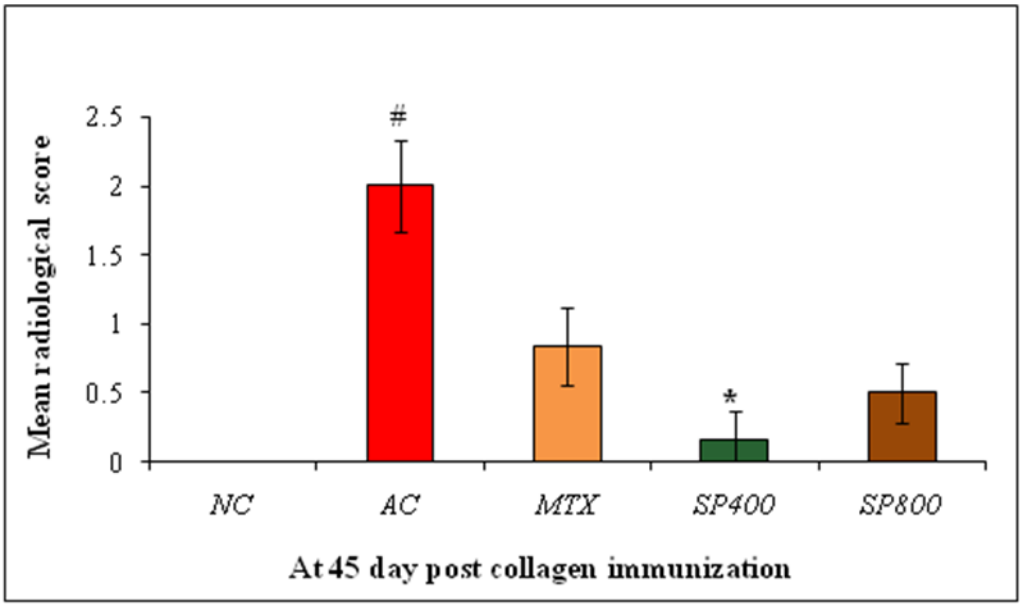


Figure: 7 represent mean radiological score at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA. *P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.

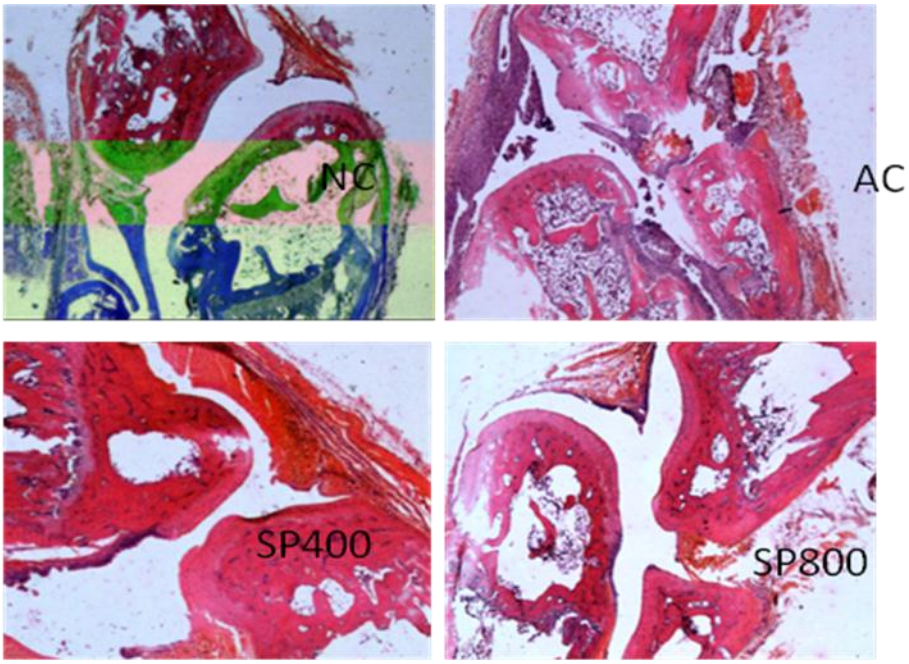
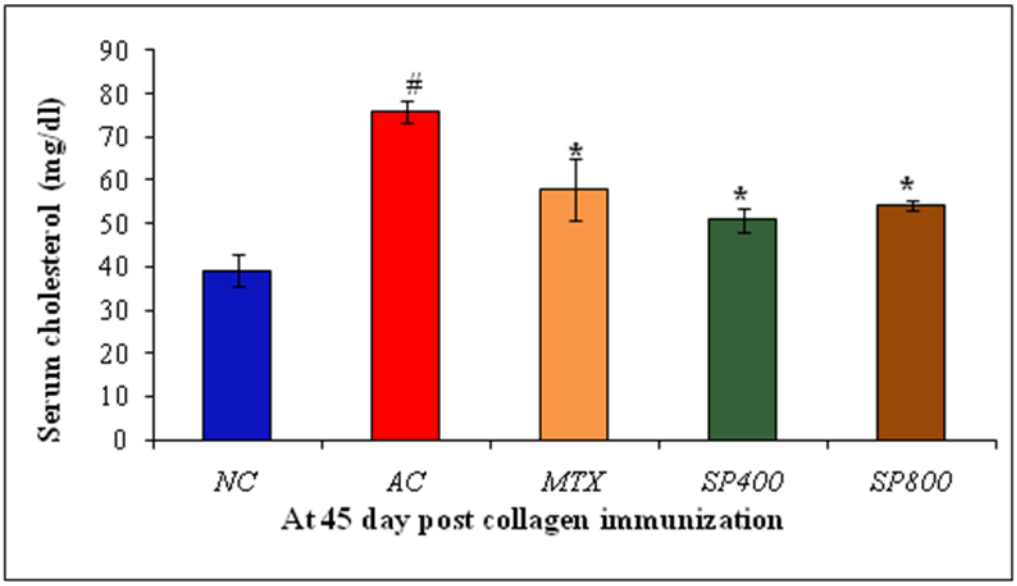


Fig. 8: Histological analysis of joints of rats. Light micrographs (2.5x10X) of rat phalangial joints (hematoxylin and eosin stained). NC = Normal control; AC = Arthritic control; *SP* 400 = *S. platensis* (400 mg kg^{-1}); *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



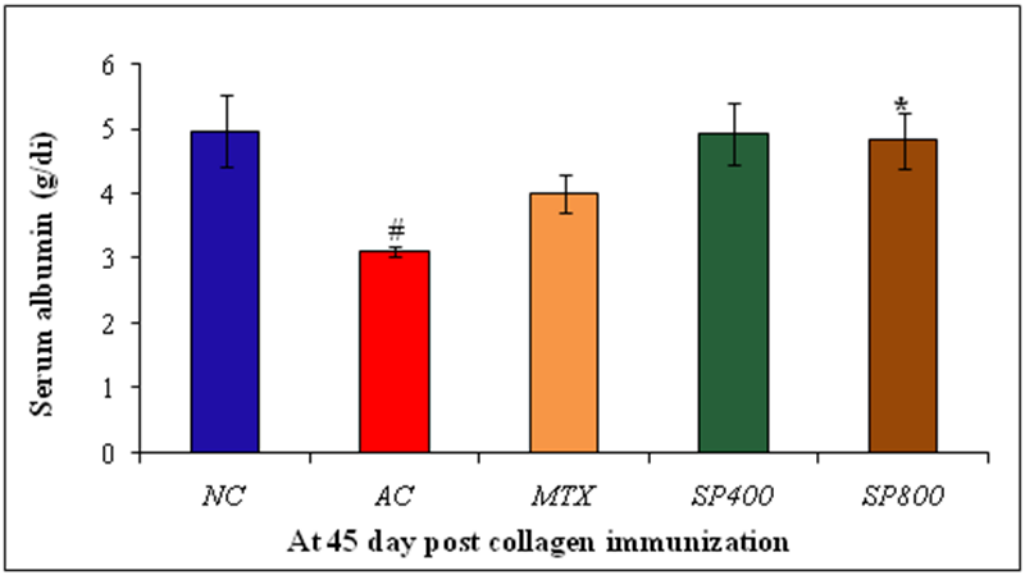
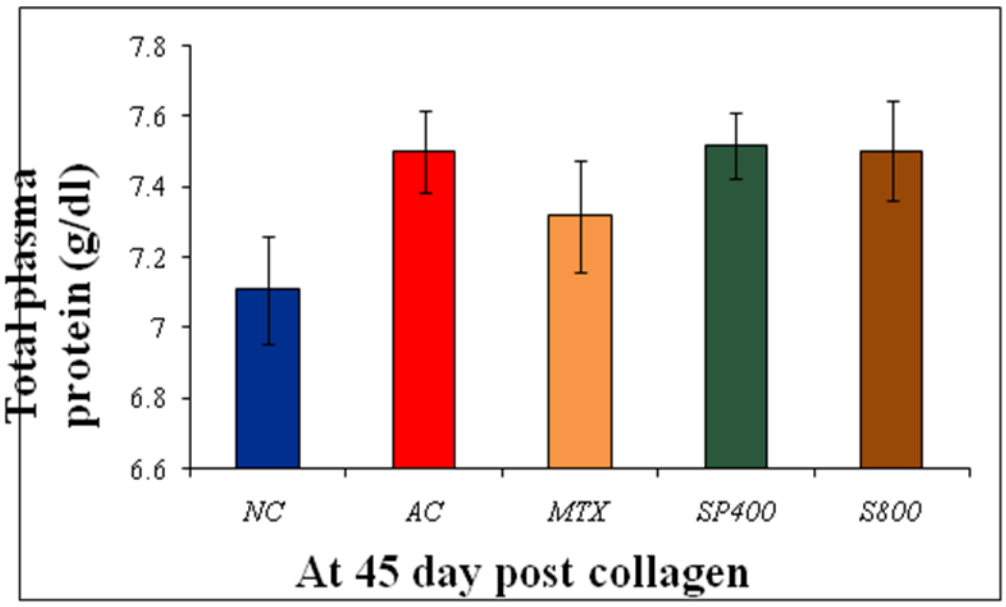


Figure: 9 represent serum cholesterol level in mg/dl of rats and serum albumin at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA.
*P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC
NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



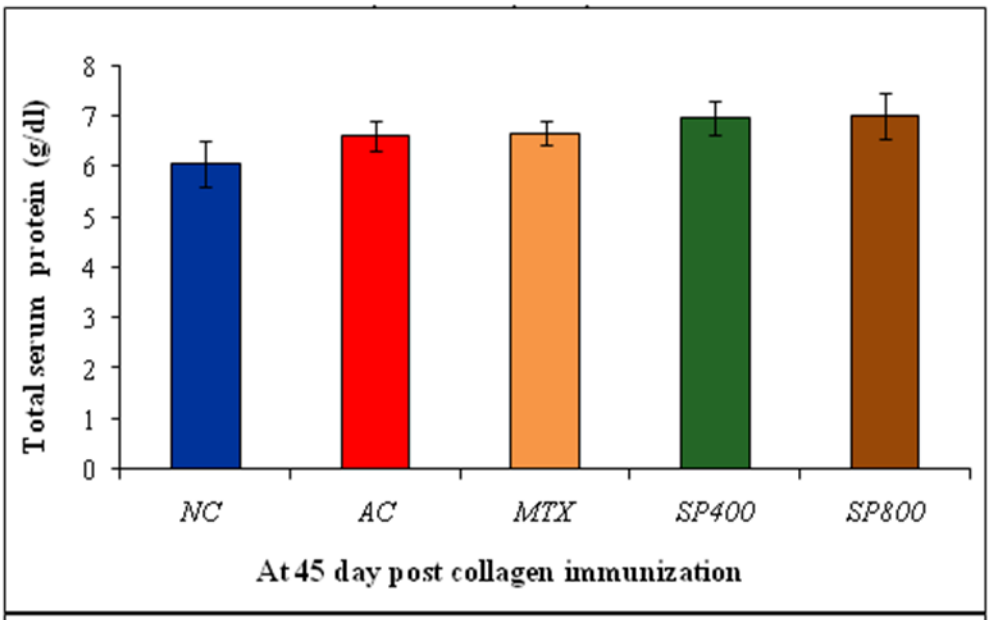
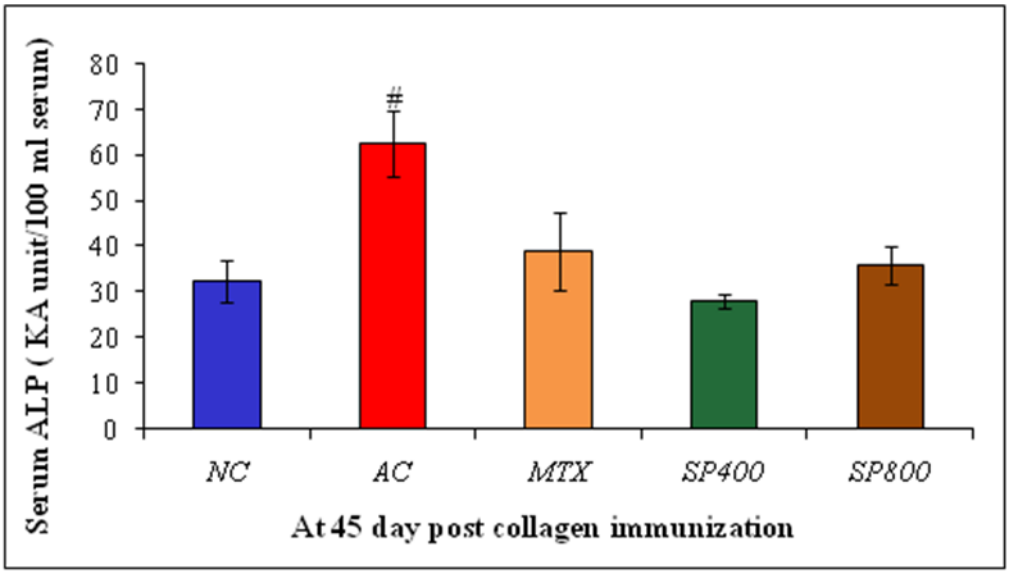


Figure: 10 represent plasma protein level of rats and serum protein level at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA.
*P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC
NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



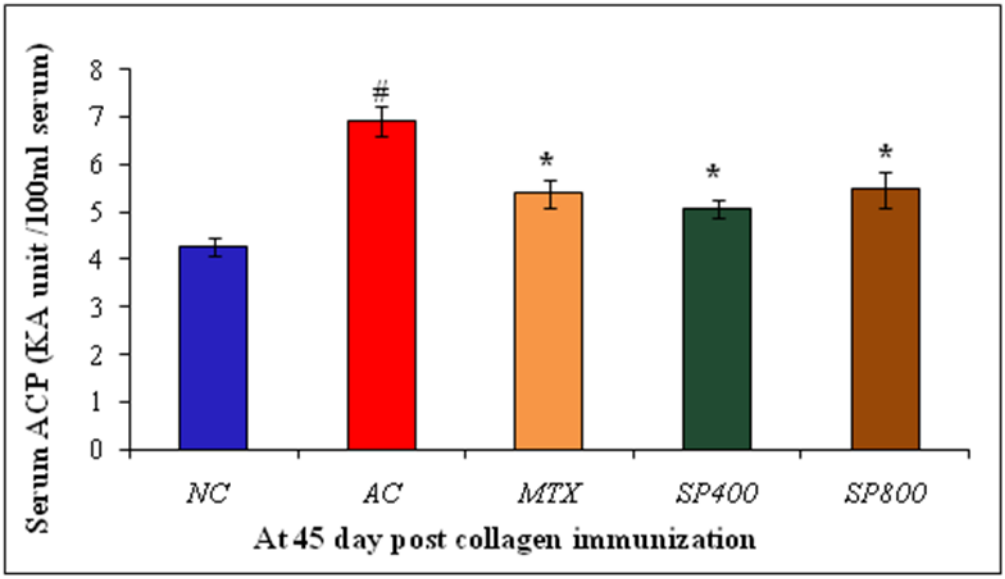
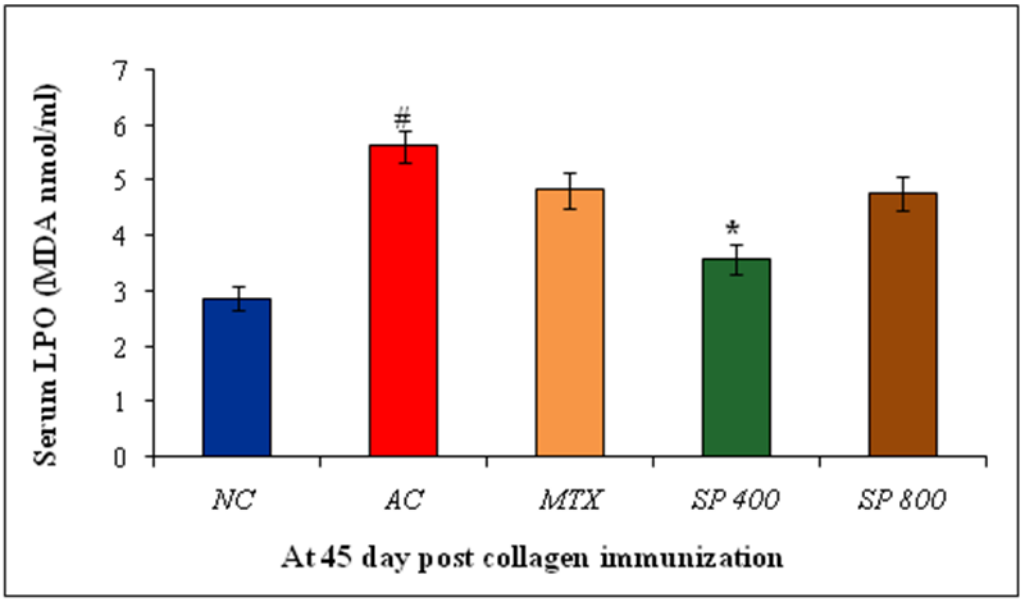


Figure: 11 represent serum ALP level and serum ACP activity of rats at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA.
*P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC
NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



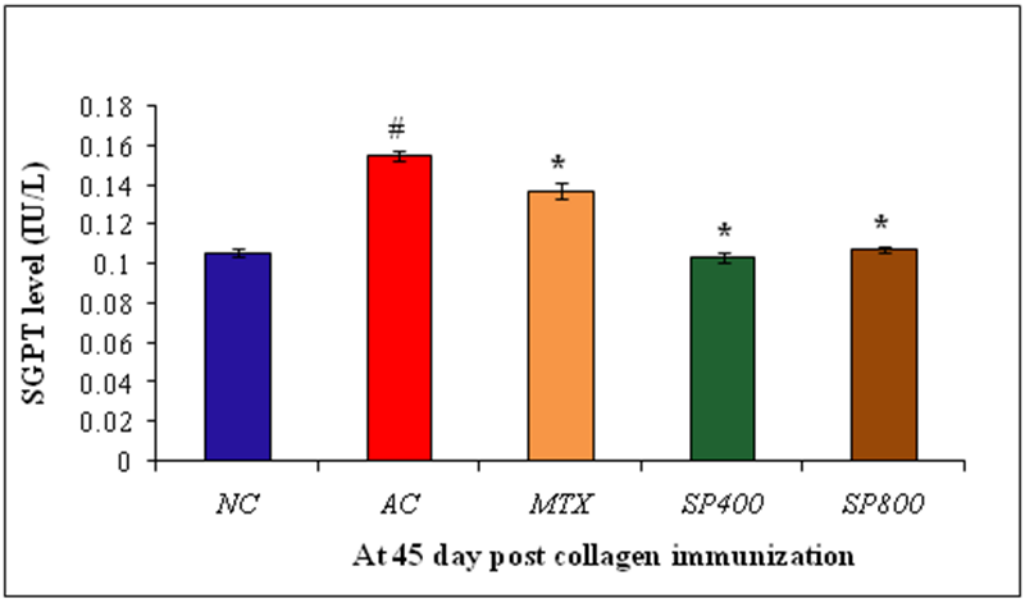
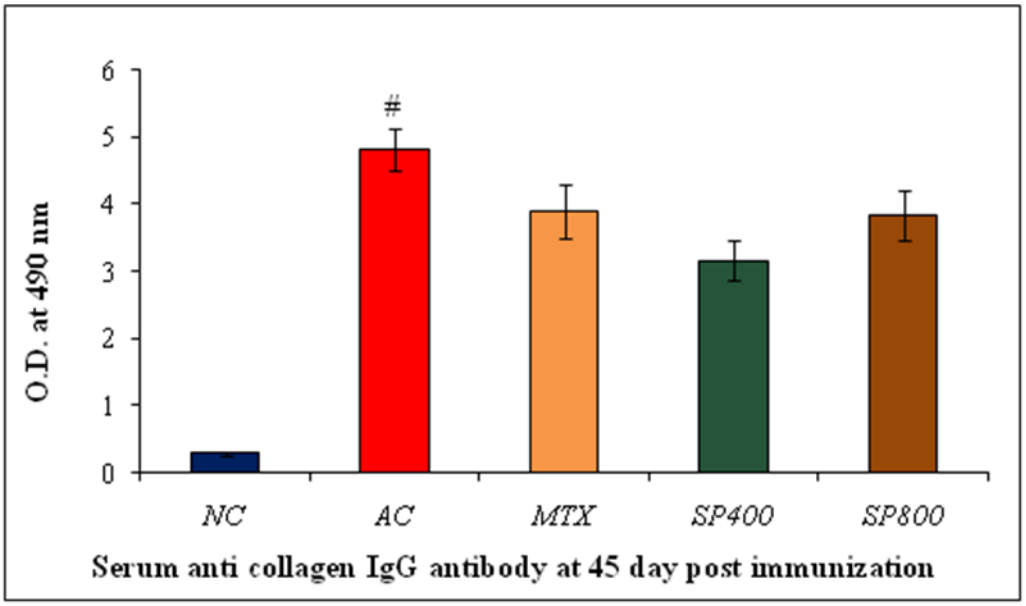


Figure: 12 represent serum LPO level and SGPT activity of rats at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA.

*P ≤ 0.05 vs AC, #P ≤ 0.05 vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



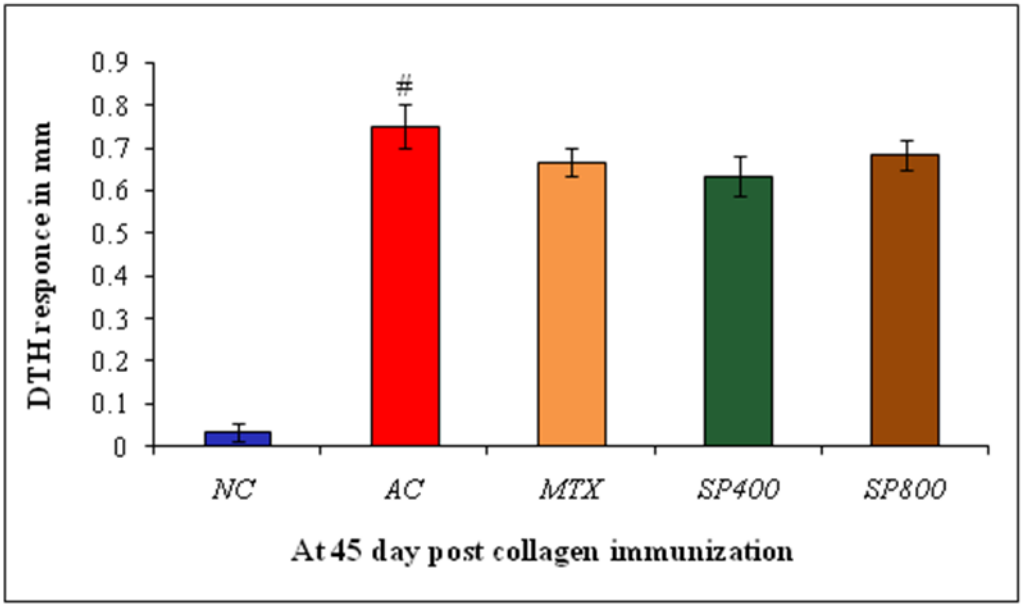
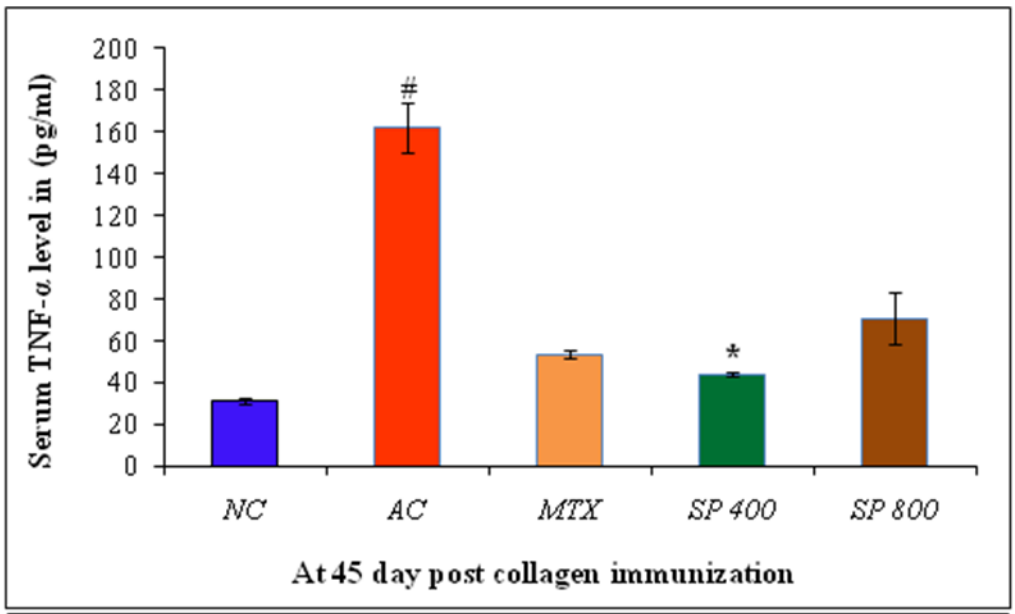


Figure: 13 represent serum anti-collagen IgG at 45 day post collagen immunization, and DTH response at 45 day. The values are expressed mean ± S.E.M., N = 6; one way ANOVA. $^{*}P \leq 0.05$ vs AC, $^{\#}P \leq 0.05$ vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.



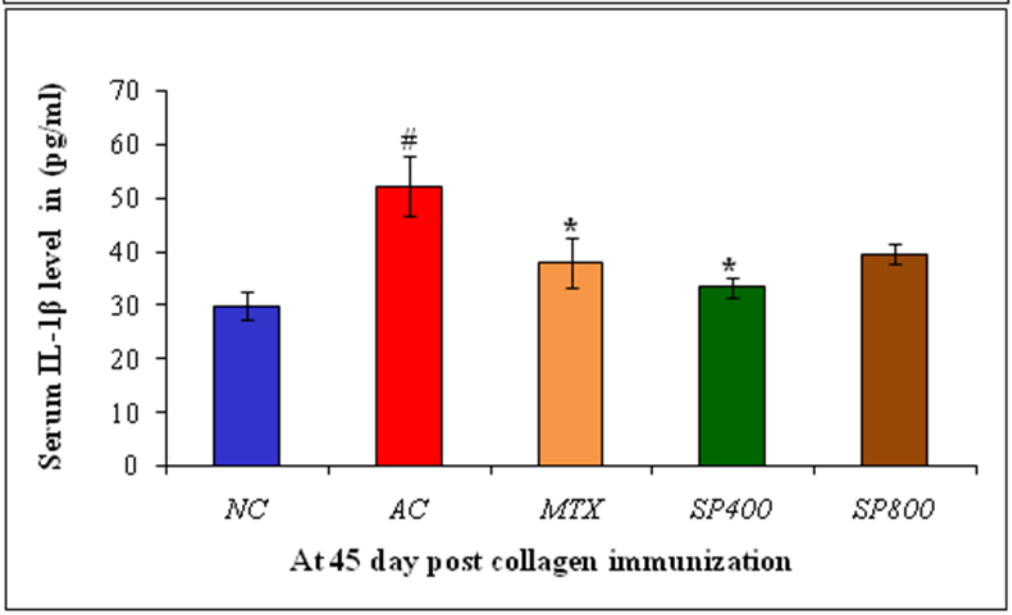


Figure: 14 represent serum TNF-α and IL-1β at 45 day post collagen immunization. The values are expressed mean ± S.E.M., N = 6; one way ANOVA. $^{*}P \leq 0.05$ vs AC, $^{\#}P \leq 0.05$ vs NC

NC = Normal control, AC = Arthritic control, MTX = Methotrexate (0.3 mg kg^{-1}), *SP* 400 = *S. platensis* (400 mg kg^{-1}), *SP* 800 = *S. platensis* (800 mg kg^{-1}) treated group.