CHAPTER 4

Studies on the anti-arthritic effect of *Bacopa monniera* (L.) Wettst

4.1 Introduction

Arthritis is a chronic, systemic disorder with symmetrical inflammatory polyarthritis, progressive joint damage and extra-articular involvement of many organs (Darlington and Stone, 2001). As indicated in the introductory chapter, the progression of arthritis occurs with the release of a wide range of pro-inflammatory mediators like eicosanoids, NO, ROS and cytokines into the synovium which operate to initiate cartilage destruction (Huet et al., 1993; Jawaheer et al, 1994; Arend and Dayer, 1995). Large amount of these mediators cause protein nitration, activation of Poly (ADP-ribose) synthetase (PARS), DNA strand breakages, inhibition of cellular metabolic pathways and signal transduction mechanisms and lipid peroxidation in the synovium which ultimately results in joint erosion and cartilage destruction (Pryor and Squadrito, 1995; Beckman et al., 1990; Portanova et al., 1996).

Lipid peroxidation is one of the underlying mechanisms of rheumatoid arthritis. During this process, peroxidation of the affected tissues cause generation of ROS such as superoxide ions \( \left( \text{O}_2^- \right) \) and hydroxyl radicals \( \left( \text{HO}^- \right) \) and non-radical species, such as hydrogen peroxide \( \left( \text{H}_2\text{O}_2 \right) \) that modifies gene expression leading to disorders of cell proliferation like loss of receptor, enzyme and transporter functions (Brown-Galatola and Hall, 1992). In arthritic patients lipid peroxidation coupled to decrease in antioxidant status cause collagen hydrolysis and activation of matrix metalloproteinases leading to degradation of extracellular matrix in the synovium and joints (Freeman and Crapo, 1982; Mapp et al., 1995; Henrotin et al., 2003).

Another characteristic feature observed during arthritis is lysosomal instability (Weissmann, 1972). Lysosomes are group of cytoplasmic organelles present in numerous animal tissues, characterized by their content of glycohydrolases.
During arthritis, the nature of the lysosomal membrane gets altered which causes lysosomes to fuse with the cell membrane and extrude an array of enzymes like β-glucuronidase, β-hexosaminidase, cathepsin D and collagenolytic enzymes. These enzymes solubilize collagen, destroy structural macromolecules of connective tissues and alters metabolism of glycosaminoglycans (GAGs) and glycoproteins causing disruption of cellular activities (Kuberasampath and Bose, 1980; Giuliani et al., 2002).

Although many infectious agents can cause inflammatory arthritis, the actual antigens behind autoimmunity are GAGs (Wang and Roehrl, 2002). Reports state that oxygen and nitrogen radicals generated during oxidative stress damage extracellular matrix and cellular elements in cartilage by upregulating mediators of matrix degradation that releases GAGs into circulation (Panasyuk et al., 1994; Heliovaara et al., 1994; Henrotin et al., 2003; Rees et al., 2003; Rees et al., 2004). Circulating or locally released GAGs then induce the clonal expansion of various GAG-binding cells like T cells, B cells and macrophages. These cells, because of their enhanced binding to GAGs, preferentially migrate and adhere to connective tissue where GAGs are abundant. GAGs expressed on endothelial and synovial lining cells then facilitate the extravasation and adherence of GAG-binding cells from the bloodstream into GAG-rich environments, such as connective tissue and cartilage (Chakrabarti and Park, 1980). Excessive and prolonged accumulation of these abnormal cells eventually leads to pathological symptoms, including damage of joint cartilage and bone erosion (Couchman, 2001).

Many experimental arthritis models have contributed to the basic understanding of joint disease and to the development of effective anti-arthritic agents (Griffiths et al., 1995). Two experimental models frequently used to study such disease processes and evaluation of possible therapeutic agents are rat adjuvant induced arthritis (AIA) and type II collagen-induced arthritis (CIA). In AIA model, the development of arthritis in rats is well defined. After complete Freund’s adjuvant induction, it is possible to distinguish between the acute stage (from day 0 to day 7) and the chronic stage (after 7th day) characterized by recurrent inflammatory bouts resulting in periarticular, articular and bone lesions (Pearson, 1956; Billingham, 1983).
Collagen-induced arthritis (CIA) in rats differs from AIA. The difference is that CIA shares many of its pathophysiological properties with human rheumatoid arthritis, such as mononuclear cell infiltration, pannus development, fibrin deposition, cartilage and bone erosion (Durie et al., 1994). Immunization of rats with type II collagen results in a complex pathogenic immune response which depends on specific MHC haplotypes (H-2^d and H-2^b) and CII-specific Th-1 type INF-γ producing T cells and B cell responses (IgG^2a producing) (Myers et al., 1997). CIA involves both humoral and cellular immune response and treatments designed to interfere with this immune response prevents the onset of CIA (Durie et al., 1994; Haqqi et al., 1996).

Today, there is a widespread interest in the use of antioxidant supplementation by patients with inflammatory arthritis, although proof of efficacy is modest. Various forms of antioxidant therapy have demonstrated promising results in experimental arthritis models (Bandt et al., 2002). A traditional Mediterranean diet relatively high in antioxidants was reported to improve disease activity and functional status compared with a standard ‘Western’ diet (Hagfors et al., 2003). Therapeutic treatment with indigenous herbal Sidha formulations like Kalpaamrutha and active plant constituents like taxol and epigallocatechin-3-gallate were also reported to counter arthritis in rats (Arsenault et al., 1998; Haqqi et al., 1999; Mythilypriya et al., 2008 a, b).

Results of the previous chapter showed that BME administration inhibited carrageenan induced 5-LOX, 15-LOX, COX, NOS and MPO activities. Thus BME exhibits anti-inflammatory effect by inhibiting multiple mediators of inflammation. Inhibition of these pro-inflammatory mediators is of importance as upregulation of these mediators can cause progression of chronic inflammatory diseases like arthritis (Crofford, 1994; Jang and Murrel, 1998; Taysi et al., 2002; Giuliani et al., 2002). The Ayurvedic texts have pointed out the use of *Bacopa monniera* in the treatment of rheumatism, but so far in literature no detailed report is available regarding the anti-arthritic effects of this herb. Hence, the present study was undertaken to study the effect of BME in modulating inflammation, oxidative stress and lysosomal instability during experimentally induced arthritis. Adjuvant
induced and type II collagen induced arthritis in rats were used as model systems. Indomethacin, a non-steroidal anti-inflammatory drug was used as positive control. The results of these investigations are presented and discussed in this chapter.

Section 1

Effect of BME on adjuvant induced arthritis in rats

4.2 Materials and Methods

Arthritis was induced in rats by injecting 0.1 ml of complete Freund’s adjuvant (CFA) into the right hind paw of Wistar rats. A preventive regimen (pre-treatment of rats with BME (100 mg/kg) for 7 days prior to arthritic induction) and therapeutic regimen (treatment of rats with BME from 3rd day post-arthritic induction) were carried out to evaluate the efficacy of BME. Indomethacin (3 mg/kg) was used as positive control. The treatment in each group was continued till day 30. The details of all the experimental procedures conducted are given in Chapter 2.

4.3 Results

4.3.1 Effect of BME on paw volume in AIA rats

Figure 4.1 shows the changes in paw volume in control and experimental animals. Paw edema and redness developed over the time period and reached constant at the end of two weeks. In BME administered group (preventive regimen), the disease was attenuated as evident by significant paw edema inhibition on day 28. Indomethacin treated group also showed significant decrease in paw edema.

It was found that prevention regimen was superior in alleviating arthritis compared to therapeutic regimen as the onset of arthritis was delayed. The inflammation in the digits and erythema completely subsided in these rats (fig. 4.2 c). The anti-edematogenic effect of BME was comparable with Indomethacin. A substantial decrease in paw edema was also observed in rats given therapeutic regimen. Since this mode of regimen also decreased clinical signs of arthritis in rats, this mode of treatment was followed for assaying biochemical parameters.
## Anti-arthritic property of Bacopa monniera

### 4.3.2 Effect of BME on body weight changes in AIA rats

Figure 4.3 indicates the changes in body weight of arthritic and drug-treated arthritic rats over a 28-day period. Rats administered indomethacin also showed decreased body weight gain, whereas treatment with BME prevented the decrease in body weight caused by arthritic induction. The body weight of BME-treated rats resembled that of normal rats.

Figure 4.1 Effect of BME on paw volume changes in AIA rats. (a) NC: non-immunized normal rats, (b) AIA: rats immunized with CFA, (c) Preventive: rats pre-treated with BME (100 mg/kg) prior to arthritic induction, (d) Therapeutic: rats given BME post arthritic induction, (e) INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05).

![Graph showing paw volume changes in AIA rats](image)

### 4.3.3 Effect of BME on paw volume changes after preventive treatment

Figure 4.2 Effect of BME on paw volume changes after preventive treatment. (a) NC: non-immunized normal rats, (b) AIA: rats immunized with CFA, (c) BME: rats pre-treated with BME (100 mg/kg) prior to arthritic induction, (d) INDO: immunized rats given Indomethacin (3 mg/kg).

![Images showing paw volume changes](image)
4.3.2 Effect of BME on body weight changes in AIA rats

Figure 4.3 indicates the changes in body weight of arthritic and drug treated arthritic rats during the course of experiment. A decrease in body weight in arthritic rats was observed from 2\textsuperscript{nd} week onwards. Rats administered Indomethacin also showed decreased body weight. Treatment with BME prevented the decrease in body weight caused by arthritic induction. Improvement in weight was observed from the third week onwards. At the end of 60 days, the body weight of BME treated rats resembled that of normal rats.

![Figure 4.3 Effect of BME on body weight changes in AIA rats. NC: non-immunized normal rats, ARTH: rats immunized with arthritis, BME: immunized rats given Bacopa extract (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). * Statistical significance with AIA control (p < 0.05).](image)

4.3.3 Effect of BME on serum enzymes in AIA rats

The effect of BME on serum ALP, GPT and GOT activities in arthritic and BME supplemented arthritic rats were studied. A marked increase in ALP, GOT and GPT activities were observed in the serum of arthritic rats. BME treatment significantly decreased the activities of these enzymes compared to arthritic control. However, the activities of these enzymes were found increased in Indomethacin treated group (table 4.1).
### Table 4.1 Effect of BME on activities of serum enzymes of AIA rats

<table>
<thead>
<tr>
<th></th>
<th>ALP</th>
<th>SGPT</th>
<th>SGOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>2.79 ± 0.02</td>
<td>0.45 ± 0.03</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>AIA</td>
<td>4.55 ± 0.02*</td>
<td>0.82 ± 0.04*</td>
<td>0.63 ± 0.05*</td>
</tr>
<tr>
<td>BME</td>
<td>3.11 ± 0.01*</td>
<td>0.51 ± 0.03*</td>
<td>0.38 ± 0.02*</td>
</tr>
<tr>
<td>INDO</td>
<td>3.63 ± 0.07</td>
<td>0.92 ± 0.02</td>
<td>0.62 ± 0.02</td>
</tr>
</tbody>
</table>

NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Units: GOT, x10^2 µM of pyruvate liberated/min/mg protein; ALP, x10^3 µM of phenol liberated/min/mg protein. Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). # Statistical significance with AIA control (p < 0.05).

### 4.3.4 Effect of BME on organ weight changes in AIA rats

Arthritic rats showed increase in spleen weight compared to normal control. However, there was no significant change in liver weight of arthritic rats with respect to normal control. Treatment with BME significantly reversed adjuvant induced increase in spleen weight by 30%. Indomethacin treatment brought no significant change in splenic weight compared to arthritic control (table 4.2).

### Table 4.2 Effect of BME on organ weight changes in AIA rats

<table>
<thead>
<tr>
<th></th>
<th>Spleen weight *</th>
<th>Liver weight *</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0.32 ± 0.04</td>
<td>4.1 ± 0.01</td>
</tr>
<tr>
<td>AIA</td>
<td>0.66 ± 0.03*</td>
<td>3.8 ± 0.01</td>
</tr>
<tr>
<td>BME</td>
<td>0.39 ± 0.04*</td>
<td>3.9 ± 0.02</td>
</tr>
<tr>
<td>INDO</td>
<td>0.50 ± 0.02</td>
<td>4.1 ± 0.03</td>
</tr>
</tbody>
</table>

NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). * g/100 g body weight. Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). # Statistical significance with AIA control (p < 0.05).
4.3.5 Effect of BME on lipid peroxidation in liver and spleen of AIA rats

The effect of BME on lipid peroxidation in arthritic rats was studied by assaying MDA levels in liver and spleen. Adjuvant induction significantly increased the concentration of MDA in the liver and spleen of arthritic rats. Treatment with BME significantly decreased MDA levels in the liver and spleen tissue compared to arthritic rats (fig. 4.4). Indomethacin administration also showed similar effect in the spleen.

![Figure 4.4 Effect of BME on lipid peroxidation in AIA rats. NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). # Statistical significance with normal control (p < 0.05). * Statistical significance with AIA control (p < 0.05).](image)

4.3.6 Effect of BME on SOD activity in liver and spleen of AIA rats

The effect of BME on antioxidant status in arthritic rats was measured by assaying the activity of SOD, an indicator against superoxide anions. A significant increase in the activity of SOD was observed in the liver and spleen of AIA rats compared to normal control (fig. 4.5). Administration of BME significantly increased SOD activity in liver compared to arthritic control. In the spleen, BME administration brought SOD activity to near normal values. Indomethacin treated group showed no significant difference in activity with arthritic group.
4.3.7 Effect of BME on GSH levels in AIA rats

Adjuvant induction significantly decreased the GSH level in liver and spleen of arthritic animals compared to normal group. BME treatment significantly improved the level of GSH in the spleen and liver (table 4.3). Indomethacin treatment did not bring about any change in GSH levels with respect to arthritic control.

Table 4.3 Effect of BME on GSH levels in AIA rats

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>AIA</th>
<th>BME</th>
<th>INDO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td>500 ± 8.5</td>
<td>360 ± 4.9*</td>
<td>483 ± 8.1*</td>
<td>373 ± 7.6</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>150 ± 7.8</td>
<td>89 ± 2.3*</td>
<td>132 ± 2.9*</td>
<td>92 ± 2.5</td>
</tr>
</tbody>
</table>

NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). 1 unit of enzyme activity is the enzyme concentration required to inhibit the chromogen production by 50% in 1 min under assay conditions. Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p<0.05). # Statistical significance with AIA control (p < 0.05).
4.3.8 Effect of BME on β-glucuronidase and β-hexosaminidase activities in liver and spleen of AIA rats

Table 4.4 and 4.5 shows the effect of BME on the activities of lysosomal enzymes in the liver and spleen of arthritic rats. In AIA induced rats, the activities of β-glucuronidase and β-hexosaminidase were significantly increased when compared to control rats. Administration of BME to arthritic rats significantly decreased the activities of these enzymes with respect to arthritic control. Indomethacin treatment did not bring about any change in these enzyme activities with respect to arthritic control.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>AIA</th>
<th>BME</th>
<th>INDO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver (U/mg protein)</strong></td>
<td></td>
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<tr>
<td></td>
<td>25.2 ± 0.81</td>
<td>40.5 ± 1.3*</td>
<td>31.8 ± 1.1*</td>
<td>39.3 ± 1.8</td>
</tr>
<tr>
<td><strong>Spleen (U/mg protein)</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>29.8 ± 0.81</td>
<td>49.3 ± 1.1*</td>
<td>36.3 ± 0.9*</td>
<td>48.5 ± 0.9</td>
</tr>
</tbody>
</table>

NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). # Statistical significance with AIA control (p < 0.05). $ \text{p-nitrophenol liberated per hour per g protein.}$

Table 4.5 Effect of BME on β-hexosaminidase activity in AIA rats

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>AIA</th>
<th>BME</th>
<th>INDO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver (U/mg protein)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.3 ± 0.83</td>
<td>39.9 ± 1.1*</td>
<td>29.4 ± 1.6*</td>
<td>35.3 ± 1.3*</td>
</tr>
<tr>
<td><strong>Spleen (U/mg protein)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.4 ± 0.71</td>
<td>32.3 ± 7.4*</td>
<td>23.8 ± 0.7*</td>
<td>29.4 ± 0.5*</td>
</tr>
</tbody>
</table>

NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). # Statistical significance with AIA control (p < 0.05). $ \text{p-nitrophenol liberated per hour per g protein.}$

4.3.9 Effect of BME on hyaluronidase, collagenase and cathepsin D activities in cartilage of AIA rats

The effect of BME on the activities of hyaluronidase, collagenase and cathepsin D were studied in cartilage extract of arthritic rats. Arthritis induction significantly increased the activities of hyaluronidase, collagenase and cathepsin D
in the cartilage of AIA rats. Treatment with BME significantly decreased the activities of hyaluronidase, collagenase and cathepsin in cartilage with respect to arthritic control (fig. 4.6). Indomethacin treatment did not bring about any change in the activities of hyaluronidase, collagenase and cathepsin with respect to arthritic control.

![Figure 4.6](image)

**Figure 4.6 Effect of BME on hyaluronidase, collagenase and cathepsin D activities in cartilage of AIA rats** NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 4). Units: hyaluronidase, Cathepsin D, collagenase. Results are expressed as mean ± SEM (n = 6). # Statistical significance with normal control (p<0.05). * Statistical significance with AIA control (p<0.05).

## 4.3.10 Effect of BME on GAG levels in articular cartilage of AIA rats

The effect of BME on GAG levels in cartilage tissue of arthritic rats was studied. Arthritic induction significantly decreased GAG levels in articular cartilage of rats. In BME administered rats, the GAG level in the articular cartilage was significantly higher compared to arthritic control (table 4.6). Indomethacin treatment increased the level of GAG in articular cartilage, but the effect was not significant.
Table 4.6 Effect of BME on GAG levels in articular cartilage of AIA rats

<table>
<thead>
<tr>
<th></th>
<th>GAG g</th>
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<tbody>
<tr>
<td>NC</td>
<td>188.4 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>AIA</td>
<td>153.0 ± 2.7 *</td>
<td></td>
</tr>
<tr>
<td>BME</td>
<td>178.5 ± 2.5 *</td>
<td></td>
</tr>
<tr>
<td>INDO</td>
<td>158.9 ± 2.8</td>
<td></td>
</tr>
</tbody>
</table>

NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 4). $\mu g$ chondroitin sulphate released /mg defatted cartilage. Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p<0.05). * Statistical significance with AIA control (p < 0.05).

4.3.11 Effect of BME on PGE$_2$ levels in paw tissue of AIA rats

The effect of BME on PGE$_2$ levels in paw tissue of arthritic rats was studied. Arthritis induction significantly increased the level of PGE$_2$ in the paw tissue of arthritic rats. Treatment with BME significantly decreased the level of PGE$_2$ in the paw tissue of arthritic rats (fig. 4.7). Rats administered with Indomethacin also showed significant decrease in PGE$_2$ in comparison with arthritic control group.

![Figure 4.7 Effect of BME on PGE$_2$ production in AIA rats. NC: non-immunized normal rats, AIA: rats immunized with CFA, BME: immunized rats given BME (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 4). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p<0.05). * Statistical significance with AIA control (p < 0.05).](image)
Section 2

Effect of BME on type II collagen induced arthritis in rats

4.4 Materials and Methods

Collagen induced arthritis was induced in rats by injecting Type II collagen (from bovine sternum) in complete Freund’s adjuvant into the base of rat tail of male adult Wistar rats (160-190 g). The drugs BME (100 mg / kg) and Indomethacin (3 mg / kg) were administered from day 14 onwards. The duration of experiment was 60 days. Details of the experimental procedure are given in Chapter 2.

4.5 Results

4.5.1 Effect of BME on arthritic index in CIA rats

Time course of the inflammatory symptoms is given in figure 4.8. The first signs of CIA developed between 11 and 13 days, as seen by an increase in paw volume and clinical score. The disease was progressive, with joint recruitment following the same pattern: tarsal, metatarsophalangeal and then interphalangeal. Treatment with BME exerted a significant attenuation in the incidence of CIA. Both the mean arthritis severity score viz. swelling in the digits and the change in hind paw diameter were found decreased with respect to arthritic group (table 4.7). Administration of Indomethacin to arthritic rats suppressed the edema during the first half of the experiment.

| Table 4.7 Effect of BME and Indomethacin on polyarthritic index |
|---------------------------------|-----|-----|-----|-----|-----|
| | Day 14 | Day 21 | Day 28 | Day 45 | Day 60 |
| ARTH | 10 ± 1.2 | 10 ± 1.1 | 11 ± 1.2 | 11 ± 1.1 | 12 ± 0.9 |
| BME | 10 ± 1.1 | 10 ± 1.2 | 8 ± 1.2* | 6 ± 0.9* | 6 ± 0.9* |
| INDO | 10 ± 1.1 | 10 ± 1.1 | 7 ± 1.1* | 7 ± 0.9* | 7 ± 0.8* |

Arthritis was induced by intradermal injection of CII in CFA into the base of rat tails. BME (100 mg/kg) and INDO (3 mg/kg) were given intragastrically from d14 to d60 after booster immunization. Polyarthritic index was evaluated at d14, d21, d40 and d60 as described in the Methods. Results are expressed as mean ± SEM (n = 6). * Statistical significance with ARTH control (p < 0.05).
Figure 4.8 Effect of BME on progression of established CIA in rats. NC: non-immunized normal rats, ARTH: rats immunized with arthritis, BME: immunized rats given Bacopa extract (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). # Statistical significance with AIA control (p < 0.05).

4.5.2 Effect of BME on 5-lipoxygenase activity in mononuclear cells of CIA rats

To study effect of BME on 5-lipoxygenase activity in mononuclear cells, blood was collected from the retroorbital vein of arthritic rats on day 60. The cells were isolated from rat blood by density gradient centrifugation using histopaque and lysed by freeze-thaw cycles. Normal rats showed very low activity of 5-lipoxygenase activity. Induction of arthritis using type II collagen significantly increased the activity of 5-lipoxygenase in rat blood mononuclear cells compared to cells isolated from normal rats.

Treatment with BME significantly decreased the activity of 5-lipoxygenase in mononuclear cells with respect to arthritic control (fig. 4.9). Treatment with Indomethacin did not show any significant change in 5-lipoxygenase activity with respect to arthritic rats.
4.5.3 Effect of BME on cyclooxygenase activity in paw tissue of CIA rats

To evaluate the activity of cyclooxygenase, paw tissue was isolated. Normal rats showed very less activity of cyclooxygenase in paw tissue. Arthritis induction significantly increased total cyclooxygenase activity in the paw tissue compared to normal control paws. Treatment with BME and Indomethacin significantly decreased the activity of total COX in paw tissue with respect to arthritic control (fig. 4.10 A).

To evaluate the % COX-2 inhibition, a colorimetric assay kit employing SC-560 as COX-2 inhibitor was used. COX-2 and COX-1 inhibition percentages are shown in figure 4.10 B. Paw tissues from BME treated group showed 89% COX-2 inhibition and 11% COX-1 inhibition. Indomethacin treated group exhibited 11% COX-2 inhibition and 89% COX-1 inhibition.
4.5.4 Effect of BME on myeloperoxidase activity in articular cartilage of CIA

The effect of BME on MPO activity was studied in cartilage tissue of arthritic rats. Arthritis induction significantly increased the activity of MPO in cartilage tissue. Treatment of arthritic rats with BME significantly decreased the activity of MPO in the cartilage with respect to arthritic control (fig. 4.11).
Indomethacin treatment also significantly decreased MPO activity in the articular cartilage with respect to arthritic control.

**Figure 4.11** Effect of BME on MPO activity in articular cartilage of CIA induced rats. NC: non-immunized normal rats, ARTH: rats immunized with arthritis, BME: immunized rats given Bacopa extract (100 mg/kg), INDO: immunized rats given Indomethacin (3 mg/kg). Results are expressed as mean ± SEM (n = 6). * Statistical significance with normal control (p < 0.05). # Statistical significance with AIA control (p < 0.05).

### 4.5.5 Effect of BME on β-glucuronidase and β-hexosaminidase activities in cartilage of CIA rats

Activities of β-glucuronidase and β-hexosaminidase were very low in cartilage tissue of normal rats. Arthritis induction significantly increased the activities of β-glucuronidase and β-hexosaminidase in the articular cartilage of rats compared to normal control.

BME treatment significantly decreased the activities of β-glucuronidase and β-hexosaminidase enzymes in the cartilage tissue with respect to arthritic control (fig. 4.12). Indomethacin treatment also decreased the activities of these enzymes but the effect was not significant.
4.5.6 Effect of BME on nitric oxide synthase activity in synovial effusate of CIA rats

The effect of BME on nitric oxide synthase activity was studied in synovial effusate of arthritic rats. Normal rats showed very less activity of nitric oxide synthase in synovial effusate. Arthritis induction significantly increased the activity of nitric oxide synthase in synovial effusate of rats compared to normal control.

BME treatment showed a change in nitric oxide synthase activity with arthritic control but the effect was not significant. Rats administered with Indomethacin treatment also showed no significant change in the activity of nitric oxide synthase with arthritic control (fig. 4.13).
4.5.7 Effect of BME on anti-CII antibody production in serum of CIA rats

The serum levels of CII-specific antibodies like IgM and IgG were evaluated by ELISA. Normal rats showed very less levels of anti-CII antibodies in serum. Arthritis induction using type II collagen significantly increased level of CII-specific IgM antibody in the serum of arthritic rats on day 21. BME administered rats showed a dramatic reduction of CII-specific IgM antibodies in serum compared with arthritic control.

The levels of anti-CII IgG antibody in different groups were measured on day 60. Arthritis induction significantly elevated the level of IgG on day 60. BME supplementation significantly decreased the level of CII specific IgG antibody in serum compared to arthritic control (fig. 4.14). Indomethacin treatment also lowered the levels of IgM and IgG on day 21 and day 60 but the effect was not significant.
4.5.8 Effect of BME on histological changes in joints of CIA rats

The histopathological data of the joint tissues of control and experimental rats is given in figure 4.15. The results are of the data are also presented.
Figure 4.15 Light microscopic appearance of joint sections

Normal control
Section of normal ankle joint synovium (arrow)

Arthritic control (CIA)
The architecture of the joint is disrupted. Deeply stained cells indicate inflammatory cell infiltration. Pannus formation and hyperplasia is evident.

CIA + BME
Section of joint showing less hyperplasia, less inflammatory cell infiltration and pannus formation

CIA + INDO
Section of joint showing mild signs of hyperplasia, less inflammatory cell infiltration and pannus formation.
4.6 Discussion

In the previous chapter, the anti-edematogenic effect of BME and its ability to inhibit pro-inflammatory mediators was demonstrated. The present chapter evaluates the anti-arthritic potential of BME using two experimental model systems- adjuvant induced and type II collagen induced arthritis. In the present study, arthritis induction significantly increased the arthritic index and decreased body weight compared to normal rats. BME administration significantly decreased the clinical signs of this disease, improved body weight and arthritic index compared to arthritic control rats. BME suppressed paw swelling in the chronic phase of the disease (2nd week onwards) and inhibited the increase in the arthritis score from day 28. The hypertrophy of spleen seen in arthritic rats was also reversed on treatment with BME indicating protection.

In the present study, serum GPT, GOT and ALP activities were also found increased in arthritic group compared to normal control. Kataoka et al. (2002) reported that increased activities of these enzymes indicate chronic inflammatory state. It was also reported that during arthritis, ALP elicited the formation of bradykinin which induced bone erosion and periarticular osteopenia (Myles Glen et al., 1965; Niino-Nanke et al., 1998; Rehman and Lane, 2001). Administration of BME significantly decreased the activities of ALP, GOT and GPT in the serum showing decreased inflammatory state and toxicity indicating protection.

It has been reported that lipid peroxidation (Gupta et al., 1992) and decreased antioxidant status (Hassan et al., 2001) are underlying reasons for the progression of arthritis. In the present study, increased level of MDA coupled to decreased levels of GSH was observed in the liver and spleen of arthritic rats. This data is in concurrence with reports by Geetha et al. (1998) and Sabina and Rasool (2007) which stated increased lipid peroxidation and decreased antioxidant status in tissues of arthritic rats. Treatment with BME significantly reduced MDA levels in liver and spleen indicating attenuation of injury in these tissues. GSH, a predominant low molecular weight thiol in the cytoplasm is indispensable for protection of tissues against lipid peroxidation during arthritis (Naganuma et al., 1990; Suleimanova et al., 1992). The level of GSH was significantly decreased in
arthritic rats indicating greater utilization of this endogenous antioxidant. BME treatment blunted the depletion of GSH in the liver and spleen probably while scavenging free radicals and this action might have helped to preserve the integrity of cellular membranes in BME treated rats.

In contrast to the status of GSH, SOD activity was found enhanced in liver and spleen of arthritic control. Marklund et al. (1987) reported that during inflammation, increased delivery of NADPH from stimulated HMP shunt causes activation of plasma SOD. In the present study, BME administration increased hepatic SOD activity and returned splenic SOD activity to near normal values. The increase in hepatic SOD activity on BME administration can be regarded as a protective measure taken by liver against the devastating effects of intracellular oxygen free radicals produced during the course of arthritis progression (Kasama et al., 1988). Previous experimental studies and report from our lab have shown that Bacopa extract improved SOD activity in brain and liver of rodents induced with morphine and nicotine (Bhattachaarya et al., 2000; Sumathi et al., 2002; Vijayan and Helen, 2007). Pawar et al. (2001) reported that Bacopa monniera extract inhibited superoxide formation in polymorphonuclear cells. These evidences suggest that Bacopa monniera extract has the potential to modulate SOD activity during oxidative stress and the differences in SOD status in different tissues can be regarded as mechanisms to with stand this devastating condition.

Another characteristic feature observed in arthritis was lysosomal instability due to the release of lysosomal enzymes into extracellular compartments (Weissmann, 1972). In the present study, the activities of glycohydrolases like β-glucuronidase and β-hexosaminidase in the liver and spleen as well as activities of cathepsin D, collagenase and hyaluronidase in the cartilage increased on arthritis induction. Treadwell (1965) reported that on sensitization, liver kupffer cells extruded glycohydrolases elevating its plasma levels. Anderson (1976) demonstrated that increased edema of hind paw after arthritic induction paralleled elevated extracellular activities of lysosomal enzymes. The activities of these enzymes were also reported to be elevated in chondrocyte supernatants and serum of RA patients (Ortutay et al., 2003; Shikhman et al., 2000; Berenbaum et al.,
2000). Previous reports have stated that increased activities of hyaluronidase, cathepsin D and collagenase during experimentally induced arthritis contributed to proteolytic cleavage of extracellular matrix components leading to traumatic injury, degradation of structural macromolecules in connective tissue and cartilage proteoglycans like hyaluronic acid, the central axis of proteoglycans (Kuberasampath and Bose, 1980; Safina et al., 1992; Oranskey et al., 1973; Mort and Billington, 2001; Ramprasath et al., 2006).

In the present study, administration of BME to arthritic rats significantly decreased the activities of these glycohydrolases and proteases in the lysosomal fractions of liver, spleen and cartilage. Studies have demonstrated that localization of phytochemicals within the membranes modified membrane fluidity and lipid peroxidation, stabilized lysosomal integrity, retrieved the normal fusion of lysosomes and made lysosomes more resistant to oxidative attack (Decharneux et al., 1992; Arora et al., 2000; Middleton et al., 2000; Khopde et al., 2001; Veena et al., 2006). Vijayalakshmi et al. (1997) reported that drugs which inhibit extracellular release of lysosomal enzymes exert anti-arthritic property. In this study, decrease in lysosomal enzyme activities correlated with decreased disease severity indicating protective effect. The drug may have modified the lysosomal membrane in such a way that the discharge of acid hydrolases was prevented.

A change in the levels or molecular nature of GAGs is another feature of arthritis (Engstrom-Laurent, 1985). During arthritis, the metabolism of GAGs gets altered and loss of GAG content in femoral condyles occur (Sledge, 1993; Giuliani et al., 2002). Reports have shown that high levels of GAG in synovial fluid and blood in arthritic patients correlated positively with joint destruction (Engstrom-Laurent, 1985; Couchman, 2001). In the present study, the articular cartilage tissue of arthritic rats showed decreased GAG content compared to normal control. Bezerra et al. (2004) reported that the loss of GAGs during experimentally induced arthritis was due to the devastating effects of reactive oxygen species. Another factor responsible for the observed changes in the level of GAG in articular cartilage could be increased activity of degradative enzymes like collagenase, hyaluronidase, cathepsin D, β-glucuronidase and β-hexosaminidase in the synovial
Anti-arthritic property of *Bacopa monniera* effusate and cartilage. The concerted action of these proteases and glycohydrolases may have caused degradation of GAG.

In BME treated rats, the GAG content in cartilage was significantly higher compared to arthritic rats indicating protection against GAG loss. The decreased activity of degrading enzymes observed in the synovial effusate and cartilage may probably be a reason for the increased concentration of GAG in the cartilage. Mythilypriya et al. (2008b) reported that Kalpaamrutha, a polyherbal formulation protected cartilage against GAG loss. Another report showed that *Aralia cordata* conferred protection against loss of GAG in cartilage explants by inhibiting matrix metalloproteinase expression and enhancing tissue inhibitor of matrix metalloproteinases (TIMP-1) activity (Baek et al., 2006). In the present study, BME must have protected against GAG loss by inhibiting lysosomal and collagenolytic enzymes.

The role of COX-2 in experimental arthritis has been well documented (Holmdahl et al., 1985). Bergsman and Schoutens (1995) reported that COX-2 products especially PGE$_2$ was responsible for inflammation, joint destruction and bone resorption during arthritis. PGE$_2$ also stimulated expression of various genes viz. vascular endothelial growth factor, IL-1 and collagenase (Mauveal et al., 1994; Ben et al., 1995; Amano et al., 1996). It was reported that during inflammation, PGE$_2$ production was primarily the result of stabilization of COX-2 mRNA and translation mediated by PGE$_2$ receptor 4 (EP4) and p38 MAPKs which were induced by inflammatory stimuli (Faour et al., 2001). In the present study, increased total COX activity was accompanied by increased PGE$_2$ production in paw tissue of arthritic rats. BME treatment significantly decreased COX activity and PGE$_2$ production in the paw tissue compared to arthritic control. 89% COX-2 inhibition was observed in the paw tissue of BME treated rats. Wallace et al. (1999) reported that suppression of COX-2 alone is insufficient to produce full range of anti-inflammatory effects as COX-1 also contributes to inflammation. A recent report by Rosche et al. (2009) reported that stabilized rice bran extracts exerted anti-inflammatory effect by inhibiting COX-2 as well as to an extent COX-1 enzyme activity. BME significantly decreased COX-2 and to a lesser extent COX-1 as well
as PGE$_2$ production in paw tissues of arthritic rats. Thus the overall anti-arthritis effect exerted by BME could be primarily due to inhibition of COX and PGE$_2$ production.

NSAIDs are largely used for the treatment of rheumatoid arthritis, although they have gastric and renal toxicities (Schuna and Megeff, 2000). But prolonged usage of NSAIDs enhances articular disorders by inhibiting GAG synthesis (Anastassiades et al., 1998). In the present study, Indomethacin inhibited paw swelling, total COX activity and reduced inflammation in joint tissue as evident from histopathological studies. Fries et al. (1990) demonstrated that use of NSAID caused hepatotoxicity. Our results are in accordance with this data as the activity of ALP in the serum of Indomethacin treated rats was high indicating toxicity. The body weight of Indomethacin treated rats decreased and the hypertrophy of the spleen was also not reversed. Bisgaard (1984) and Asako et al. (1992) stated that NSAID-induced COX inhibition caused shunting of arachidonic acid to the 5-LOX pathway causing increased leukotriene production that triggered leukocyte adherence to gastric microvessels causing mucosal injury.

Role of LOX pathway and leukotrienes in aggravating arthritis has been well demonstrated (Moilanen, 1994; Gursel et al., 1997). Ahmadzadeh and colleagues (1991) reported that leukotrienes levels in synovial fluid were higher in patients with rheumatoid arthritis. The leukocyte levels, immune complexes and rheumatoid factor in synovial fluid correlated directly with LTB$_4$ levels in these patients (Ahmadzadeh et al., 1991). In the present study, arthritis induction significantly increased the activity of 5-LOX in blood mononuclear cells. Administration of BME significantly decreased this arthritis induced 5-LOX activity. Studies using different leukotriene inhibitors in animal models have shown that LTB$_4$ inhibitors have a clear effect on collagen-induced arthritis (Griffiths et al., 1995). Recently, Dhanasekaran et al. (2007) reported that Bacopa monniera extract inhibited lipoxygenase activity in brain of Alzheimer's disease induced rats. Previous studies from our lab showed that BME is an active inhibitor of Ca-A23187 stimulated 5-LOX and 15-LOX activities in mononuclear cells (Viji and Helen, 2008). These studies provide evidence that Bacopa monniera
extract has lipoxygenase inhibition potential pointing out the beneficial effects of the herb.

In this study, balanced dual inhibition of both COXs and LOX-metabolic pathways indicate advantageous pharmacological profile of BME. It is possible that the anti-arthritic effect of BME obtained may be partly related to its ability to reduce formation of COX products and LOX products, although the mechanism of action underlying this process is complex.

Nitric oxide synthase activity was found significantly increased in the synovial effusate of arthritic group. BME administration lowered the activity of this enzyme but the effect was however not significant. Similar effect was shown by Indomethacin. Perhaps decreased concentration of nitric oxide synthase inhibitory plant principles in BME may account for this observation. We presume that further concentration of anti-inflammatory principles in BME and administration of that fraction to arthritic rats would yield better results.

Reports have stated that neutrophil infiltration is a characteristic event in arthritis. It was found that neutrophil counts were elevated in synovial fluid of rheumatoid arthritic patients. These cells secrete proteases that contribute to destruction of cartilage and related joint structures (Penidoc et al., 2006). BME treatment significantly decreased myeloperoxidase activity in cartilage of arthritic rats indicating decreased neutrophil infiltration. Decreased neutrophil infiltration was also confirmed using histopathological data. Less inflammatory cell infiltration with mild hyperplasia and decreased pannus formation was observed in BME administered rats compared to arthritic control. These data indicate that BME effectively suppresses the chronic phase of arthritis, that is, the period when maximum joint destruction occurs.

In the present study, anti-CII antibodies like IgM and IgG were found elevated in serum of arthritic rats compared to normal control. Combe et al. (1997) reported a relationship between autoantibody titre and bone damage. Previous reports have demonstrated that heat-killed *Mycobacterium tuberculosis* in complete Freund’s adjuvant leads to predominant Th\(^1\) response that induces the activation of CII-specific cellular immunity and the production of CII-specific
IgG\textsuperscript{2a} and IgG\textsuperscript{2b} antibodies resulting in bone damage (Holmdahl et al., 1990; Feldmann et al., 1996; Kageyama et al., 1998; Zhang et al., 2008). Nandakumar and Holmdahl (2006) stated that the pathologic nature of elevated antibody levels was due to direct binding of these pathological antibodies to their respective antigens, immune complex formation, deposition and finally activation of complement and Fc receptors. From the data obtained, significant difference in IgM and IgG titres between arthritis and BME treated arthritic rats on day 21 and day 60 was observed. Treatment with BME significantly decreased the levels of IgG and IgM in the serum of arthritic rats. Since antibodies of IgG\textsuperscript{2a} isotype are the most efficient antibodies that activate complement (Nimmerjahn and Ravetch 2006; Zhang et al., 2008), reduced IgG antibody production as seen in BME treated group could be one of the reasons for decreased disease pathology. A correlation between the level of antibodies and clinical signs of arthritis was significant in this study indicating that rats with low levels of CII antibodies displayed less severe arthritis.

In conclusion, BME possesses remarkable anti-arthritic activity. In BME preventive regimen group, the clinical signs of arthritis were completely ameliorated in rats indicating that consumption of this extract can prevent progression of this disease. BME exerts anti-arthritic effect by inhibiting 5-LOX and COX-2 activities, increasing lysosomal stability, decreasing neutrophil infiltration, inhibiting anti-collagen antibodies, decreasing lipid peroxidation and improving antioxidant status during arthritis. At this point, it was of interest to us characterize the component(s) in BME that is responsible for mediating these pharmacological effects. For this purpose BME was purified and the effect of the purified extract in countering various inflammatory mediators associated with arthritis was studied.