Chapter 4A

(Results & Discussion)

Screening of plants for their anti-inflammatory properties and evaluation of natural compound for its anti-arthritic potency in CIA
4A.1. Introduction

Many plant products from medicinal herbs are used for the treatment and prevention of diseases. Natural plant compounds are now gaining more pharmacological attention as many unexplored plant products are showing a wide range of activities like anti-cancer, anti-inflammatory, and anti-aging (Raghav et al., 2007). Nearly 25% of all prescribed drugs are derived from plants with or without further modification, still several pharmacologically active plant-derived compounds remain unexplored (Raskin et al., 2002). Inflammation acts as a central executor in the pathogenesis of many diseases such as rheumatoid arthritis, arteriosclerosis, myocarditis, infections, cancer, metabolic disorders, and many more (Esposito and Giugliano, 2004a; Krakauer, 2004b; Ohshima and Bartsch, 1994a). Monocytes and macrophages are the key players in inflammatory responses and are also the major sources of pro-inflammatory cytokines and enzymes including tumor necrosis factor-α (TNF-α), interleukins (ILs), cyclooxygenase (COX), and nitric oxide synthase (NOS) (Bonizzi and Karin, 2004b; Duffield, 2003; Fujiwara and Kobayashi, 2005). These genes of pro-inflammatory mediators are strongly induced during inflammation and are responsible for its initiation and persistence. TNF-α and IL-1β are the cytokines that act as signaling molecules for immune cells and coordinate the inflammatory responses (Krakauer, 2004a). Cyclooxygenase-2 (COX-2) is an enzyme which is necessary for the production of pro-inflammatory prostaglandins and thus has been a target for many present anti-inflammatory and cancer-preventive drugs (Juni et al., 2005; Pathak et al., 2005). Nitric oxide (NO) is a free radical that mediates many physiological and pathophysiological processes, including neurotransmission and inflammation (Nathan and Xie, 1994a). Its formation is catalysed by three different isoforms of NOS by the conversion of L-arginine to NO and L-citrulline. Expression of the inducible isoform of NOS (iNOS) in activated macrophages is mainly responsible for production of
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pathological concentration of NO during inflammation. It is well known that nuclear factor-κB (NF-κB) plays the most important role in the immune system (Li and Verma, 2002b). NF-κB is reported to regulate the expression of nearly all inflammatory mediators involved in inflammation (Bonizzi and Karin, 2004a). Nuclear translocation of NF-κB in response to various pro-inflammatory stimuli is associated with the activation of inflammatory cascade and therefore, this transcriptional factor is a primary target of many anti-inflammatory therapeutic strategies (Li et al., 2005; Nathan and Xie, 1994c). Natural plant compounds which are able to suppress the production of inflammatory mediators from activated macrophages can act as potential anti-inflammatory agents. Therefore, this study is aimed to explore and evaluate the anti-inflammatory potential of extracts from some traditionally used medicinal herbs with unidentified anti-inflammatory activities and their mode of action. For the screening of such active components, pro-inflammatory TNF-α, IL-1β, IL-12 and enzyme-encoding genes, iNOS, and COX-2 are chosen as pro-inflammatory markers. The biological activities of the methanolic extracts against inflammation and their mode of action were examined in vitro using LPS-stimulated J774 murine macrophage cells as model.

Apart from the in vitro evaluation, assessment of anti-inflammatory agents, such as plant extracts, active natural compound is done in vivo using animal models of arthritis. Collagen induced arthritis (CIA) is an autoimmune model that in many ways resembles rheumatoid arthritis (Durie et al., 1994). It is used to address the questions of disease pathogenesis and to validate therapeutic targets. It has been widely used for preclinical testing of numerous anti-arthritic agents (Rosloniec et al., 2010). The use of animal models has contributed greatly in the generation of inflammation, cartilage destruction and bone resorption. In RA, pro-inflammatory cytokines such as TNF-α and IL-1β are abundantly expressed in the arthritic joints (Feldmann et al., 1996c). It is known that the joints of animal with CIA also showed similar observations and
blockade of these molecules results in a reduction of disease severity (Bendele, 2001a; Brennan et al., 1989). Inspite of numerous studies, which have been contributed to the understanding of various possibilities such as reactivity to cartilage proteoglycans, heat shock proteins and interactions with intestinal flora (Benslay and Bendele, 1991; Van Vollenhoven et al., 1988a), the pathogenesis for the development of inflammatory disease are not fully understood. Rheumatoid arthritis is an ‘inflammatory’ disease. Joints affected by RA often become unstable causing deformities. It is also known that when the body has any sort of inflammation, usually level of C-reactive protein (CRP) in the blood increases (Szalai, 2004). The purpose of this study was to examine whether level of CRP, a sensitive marker of disease activity in RA is associated with collagen induced arthritis in rats. The glycoproteomic analyses of this protein and other indicators of arthritis were carried out to establish co-relationship between disease severity and biochemical markers. Glycoproteomic analysis requires that a sample should be purified prior to glycan analysis (Sagi et al., 2005). To analyze a sugar containing glycoprotein of interest the sample may be enriched by affinity chromatography using lectin(s) or sugar specific ligand(s). Thus a combined glycan and protein identification approach was used to monitor and detect glycosylation changes of specific glycoproteins in CIA rat.

Natural products have served as the source of the most active ingredients of medicines since time immemorial, and now more than 80% of the pharmaceutical products are either derived from natural products or inspired by natural compounds (Harvey, 2008). Currently prescribed anti-arthritic drug regimen; nonsteroidal anti-inflammatory drugs (NSAIDs) and disease modifying antirheumatic drugs (DMARDs), can effectively manage the pain and symptoms of the disease, though with rather discouraging profile of side-effects. So, there is a pressing need for the development of novel anti-inflammatory therapeutic agents for RA that can prevent the progression of
disease and confer safe prolonged treatment. One solution to this problem is the use of alternative therapeutic approaches including herbal therapies, which have been considered safe and effective in alleviating chronic pain associated with arthritis (Soeken et al., 2003a).

*Ruta graveolens* L., (Rutaceae) commonly known as rue or Herb-of-grace, is a plant native to Mediterranean region (Raghav et al., 2006b). The aerial parts of this plant are used in folklore medicine for the treatment of disorders like rheumatism, eye ailments, dermatitis (Conway and Slocumb, 1979), etc. Up to now more than 120 compounds of different classes of natural products such as alkaloids, coumarins, flavonoids and essential oils have been identified. These classes of compounds have gained particular interest in medicinal chemistry due to their spasmyolytic, analgesic, antiphlogistic, anti-helminthic and anti-inflammatory activities. Previous work done in our lab has demonstrated the anti-inflammatory effects of Rg1 or 3-(1′-1′-dimethylallyl)-6-hydroxyl-7-methoxy-coumarin isolated from the methanolic extract of *R.graveolen’s* plant (Raghav et al., 2007). In the present report, another potential anti-inflammatory coumarin Rg3 (8-methoxy-chromen-2-one) was isolated from this plant. Further, its therapeutic potency was evaluated in the CIA rat model for its use as a possible lead compound.

In this chapter, we discuss the screening of plant extracts for their anti-inflammatory effects using cultured cells, then the development and consequent development of CIA rat models. Plasma glycoproteins from CIA rats were enriched using lectin affinity column. Glycoproteins thus obtained were subjected to difference in gel-electrophoresis (DIGE). DeCyder software was used to analyze differentially expressed protein spots and were confirmed by MALDI-TOF. Glycan part was analyzed after deglycosylation of the identified spot using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). Finally, therapeutic potency of active
compound (Rg3) was tested on the CIA rat model.

4A.2. Results

4A.2.1. Effect of plant extracts on LPS-induced NO production

Methanolic extracts of 14 different medicinal plants were screened for their anti-inflammatory activities and out of them four extracts (Grindelia robusta, Salix nigra, Arnica montana, and Quassia amara) were selected for further studies. These selected plant extracts did not exhibit any significant cytotoxic effect even at a high concentration (500 μg/ml) for 48 h of incubation, and in all cases the viability was found above 92% by MTT assay (Data not shown). Nitric oxide production by LPS-activated cells was found to be significantly inhibited (p<0.05) by all four plant extracts under study in a dose dependant manner. In the present study, selected plant extracts (100 μg/ml) were used to evaluate their anti-inflammatory activity. As shown in the Figure 4A.1 cells treated with whole plant extracts of G. robusta, S. nigra, and A. montana resulted in 2.27, 3.96, and 4.9-fold reduction, respectively in the LPS-induced NO production, while Q. amara treatment was able to reduce the same to 5.69-fold (p<0.01). L-NAME (200 μM) was used as positive control in each experiment.
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Figure 4A.1. Inhibition of nitric oxide production measured as nitrite in the cell free culture supernatants of LPS (1μg/ml) challenged J774 murine macrophages. Experimental details are as given under “Material and methods” and NO concentration in the culture supernatant was determined by Griess assay using sodium nitrite standard curve. Data are presented as means ± SD from three sets of independent experiments. *p<0.05, **p<0.01 represent significant difference compared with cells treated with LPS alone.

4A.2.2. Effect of plant extracts on LPS-induced iNOS and COX-2 protein expressions

The protein expression levels of the two major pro-inflammatory enzymes iNOS and COX-2 in LPS-challenged cells with or without the treatment of plant extract were evaluated by densitometric analysis of the Western blots (Figure 4A.2). Treatment of LPS increased the protein levels of iNOS and COX-2 in macrophage cells by 5.34 and 4.66 fold, respectively (Figure 4A.2). A significant reduction (p<0.05) in iNOS protein level was observed with all the four plant extracts and the maximum (4.4-fold, p<0.01) inhibitory effect was observed in cells treated with Q.amara extract. Similarly COX-2 protein level also showed a significant decrease in response to plant extract treatment, however, S. nigra extract showed a highly significant (2.14-fold, p<0.01) inhibitory effect in the LPS-induced COX-2 protein expression.
Figure 4A.2. Inhibition of iNOS and COX-2 enzyme levels in the J774 cells. Cells were treated with indicated concentration of extracts for 24 h. (A) Protein levels of iNOS and COX-2 in the cell lysates were determined using Western blot analysis and GAPDH was used as control. (B) Densitometric analysis of protein bands; data are presented as means ± SD from three sets of independent experiments. Experimental details are as given under “Material and methods”. *p<0.05, **p<0.01 represent significant difference compared with cells treated with LPS alone.

4A.2.3. Effect of plant extracts on pro-inflammatory cytokines

Macrophage cells treated with LPS exhibited an appreciable increase in the levels of TNF-α, IL-1β, and IL-12 in the culture supernatants (Figure 4A.3). All the four plant extracts were able to reduce the levels of TNF-α significantly in the culture supernatant and Q.amara extract exhibited highest (3.98-fold, p<0.01) inhibitory activity (Figure 4A.3a). Extracts from Q. amara and G. robusta showed highly significant (up to 2.25-fold, p<0.01) suppressive effects on the production of IL-1β by the LPS-stimulated macrophages on the contrary no significant effect was observed with A. montana extract (Figure 4A.3b). As seen in Figure 4A.3c, only A.montana and G.robusta extracts were able to inhibit the production of IL-12 in response to LPS to significant levels (2.8-fold, p<0.01 and 1.3-fold, p<0.05, respectively).
Figure 4A.3: Inhibition of pro-inflammatory cytokines production by J774 cells. Culture supernatants from treated cells were immunoassayed for TNF-α (panel A), IL-1β (panel B), and IL-12 (panel C) production by ELISA. Data are given as means of means ± SD from three sets of independent experiments. *p<0.05, **p<0.01 represent significant difference compared with cells treated with LPS alone.

4A.2.4. Effect of plant extracts on NF-κB activation

Activation of NF-κB is the key event for induction of all major inflammatory mediators. Both iNOS and COX-2 are the NF-κB inducible enzymes. Production of pro-inflammatory cytokines is also regulated by NF-κB. It was also observed that the plant extracts were able to inhibit NF-κB activation in LPS-stimulated macrophages as indicated by immunoblots (Figure 4A.4a). Densitometric analysis of the blots (Figure 4A.4b) showed that LPS stimulation radically increased NF-κB activation through the nuclear translocation of the p65 subunit of NF-κB. This phenomenon was significantly inhibited (p<0.05) in cells treated with the extracts of G. robusta, S. nigra, A. montana, or Q. amara. Among these, Q. amara showed the highest inhibition (2.08-fold, p<0.01).
nuclear translocation of NF-κB. These results were also confirmed by the immunostaining of treated cells with antibody against NF-κB p65 subunit as shown in the Figure 4A.5.

**Figure 4A.4.** Inhibition of LPS-induced nuclear translocation of NF-κB in J774 cells. Cells were treated with extracts in the absence or presence of LPS (1 μg/ml) for 30 min as described under “Material and methods”. **(A)** The levels of NF-κB, protein in the nucleus were analyzed by Western blot. This is a representative of three independent experimental set of blots; **(B)** relative densities of specific genes with the housekeeping gene β-actin. Data represent the means ± S.D.; *p<0.05, **p<0.01 compared with LPS alone.
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Figure 4A.5. Inhibition of LPS-induced nuclear translocation of NF-κB in J774 cells as visualized by immunostaining of NF-κB using FITC-conjugated antibodies. The green fluorescence indicates the location of p65 protein (magnification X 40). A representative figure from three independent sets of experiments is shown.

4A.2.5. Development of collagen induced arthritic rat model

Mild periarticular erythema, swelling of paws and ankles started within 12-14 days after the first injection of collagen. After 21 days of first injection, hind paws of the experimental rats showed significant swelling (Figure 4A.6A). We assessed the severity of the arthritis with a well established arthritic score system (Van Vollenhoven et al., 1988b). Maximum paw volume occurred around day 21 in CIA rats (data not shown).
Rats injected with acetic acid (0.05 M) were used as control group which did not show any increase in paw volume (Figure 4A.6B). Damage of cartilage and bone of the affected hind paws was observed by X-ray as shown in Figure 4A.6C while control rat was not showing any such signs (Figure 4A.6D). The paw volume in each CIA rat revealed a time-dependent increase in hind paw volume (Figure 4A.6).

![Figure 4A.6](image)

**Figure 4A.6.** Hind paw of CIA rat (A), control rat (B). X-ray image of hind limb of CIA rat (C), control rat (D). Arrows indicate the bone destruction.

4A.2.6. **Glycoprotein enrichment from plasma and gel electrophoresis and their analyses**

The proteomic studies of plasma proteins of CIA rat were carried out first by visual analysis of florescence scanned gels. Differentially expressed protein spots were observed (Figure 4A.7) in CIA rat plasma. The differential expression of these protein
spots were analyzed using DeCyder software. Total number of spots detected by DeCyder in each gel was ranging from 1500 -1700. These protein spots were uniquely numbered by synchronization to those in a reference gel. Intensity differences in spots matched between experimental and control gels were quantified, normalized to the internal standard to compare the individual protein spots between experimental and control samples. It was observed that in each gel 200 to 300 protein spots were matched to the master image. Densitometric analysis of these spots revealed 32 statistically significant spots ($p \leq 0.05$). Mass spectrometric analysis of all these 32 significant protein spots were very difficult as the spots were either not clearly visible or too small to cut from the corresponding silver stained gel. The spot number 1 and 2 as observed by DeCyder analysis were up-regulated in CIA rat plasma in comparison to that of control (Figure 4A.8). The corresponding spots from silver stained gels (Figure 4A.9) were cut and subjected to trypsin digestion followed by mass spectrometric analysis. Spot 1 and 2 were identified by MALDI-TOF-MS-peptide mass fingerprinting as C-reactive protein precursor (Figure 4A.10, Table 4A.1) and haptoglobin, respectively. The peptide sequence coverage of CRP and haptoglobin is shown in Figure 4A.10C. Monosaccharide pattern of these proteins was analyzed by HPAEC-PAD. The results revealed that both the proteins were glycosylated and fucosylation alters in CIA rat plasma. In both the proteins isolated from CIA rat plasma amount of fucose increases significantly (Table 4A.1, Figure 4A.11).
Figure 4A.7: 2D DIGE images of Con-A bound plasma protein. Cy3 labeled control rat plasma (A) and Cy5 labeled CIA rat plasma (B).

Figure 4A.8: 3-D view showing the differential expression of protein spot 1 and 2 using Decyder software.
Figure 4A.9: 2D silver stained image of Con-A bound plasma protein from control and CIA rat plasma using IPG strips pH 4-7.
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(C)

Figure 4A.10: Peptide mass fingerprinting (PMF) spectra of spot 1 (A) and spot 2 (B) from silver stained gel. (C) The sequence of spot 1 and spot 2 as available in the database. Peptides covered in PMF spectra are underlined.

Table 4A.1: Identification of differentially expressed proteins in ConA bound CIA rat plasma by MALDI-TOF MS (mowse score, Mol. Wt., Sequence coverage are as available in Swiss Prot database).

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein</th>
<th>Peptide/ matched</th>
<th>Mowse score</th>
<th>Mol. Wt. kD</th>
<th>Sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-reactive protein</td>
<td>6</td>
<td>71.4</td>
<td>25.00</td>
<td>41.7%</td>
</tr>
<tr>
<td>2</td>
<td>Haptoglobin (β chain)</td>
<td>15</td>
<td>109.1</td>
<td>45.00</td>
<td>51.5%</td>
</tr>
</tbody>
</table>
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4A.2.7. *Ruta graveolens* L. extraction for Rg3

*Ruta graveolens* methanol extract was used for the isolation of active compound, 8-methoxy-chromen-2-one (Rg3). The structure of this compound was deduced using 1H-NMR and MALDI-TOF (Raghav, 2006).

Upon isolation of the compound using RP-HPLC, this is crystallized into white needles (mg% dry wt/wt% purity by HPLC). The present study evaluated the anti-inflammatory / anti-arthritic property of this purified compound.
Figure 4A.12. (A) RP-HPLC profile of diethyl ether fraction showing nine different clear peaks (fractions) including that of the active compound Rg3. (B) Analytical HPLC profile of the isolated active compound Rg3 ensuring the purity of the compound Rg3. (C) The deduced structural formula of the active compound Rg3 and its IUPAC name of 8-methoxy-chromen-2-one (Raghav, 2006).
4A.2.8. Induction of CIA and the morphological parameters: hind paw appearance, arthritic index, arthritic score and change in body weight

Development of CIA was started with the edema and redness of the joints, which occurred approximately 2 weeks after the primary immunization with type II collagen. The difference of appearance in the healthy control, untreated and treated arthritic rats were obvious (Figure 4A.13A). Plateau of the peak of CIA response was maintained in the duration of day 25-35 with the mean AI of 113.26%, followed by a gradual decrease. Rg3 treatment of the CIA animals at both the dosages (2 mg/kg and 20 mg/kg) led to a significant (p<0.001) decrease in the severity as evident by the drastic reduction of AI soon after the treatment for 5 days (Figure 4A.13B). Towards the end of the experiment, the CIA inhibitions for both the doses of Rg3 were calculated to be 75.7% (2mg/kg) and 84.7% (20 mg/kg). Although the Rg3-treated animals were benefitted by the lowering of AI, the hind paw volumes were not totally normalized as compared to the healthy control (p<0.0001). Thus Rg3 (20 mg/kg) treatment was at par with that of indomethacin, which showed an inhibition of 87.4%.

The visual clinical assessment of the arthritic condition was expressed as arthritic score in Rg3 (2 and 20 mg/kg) groups. This was reduced significantly (P < 0.05) when compared to that of the CIA group (Figure 4A.13C).

As shown in Figure 4A.13D, treatment with Rg3 and indomethacin proved to be beneficial in the CIA associated weight loss when compared to the vehicle treated CIA rats. This suggests that Rg3 is not toxic at the given doses. Besides, Rg3 was able to positively affect the weight gain of CII-immunized rats in a dose-dependent manner. The control rats showed a gradual gain of body weight with time.
Figure 4A.13: (A) Representative photographs of hind paws from: (a) Control, (b) CIA+vehicle, (c) CIA+Rg3 (2 mg/kg/day), (d) CIA+Rg3 (20 mg/kg/day), (e) CIA + indomethacin (1 mg/kg/day). Effects of Rg3 and indomethacin treatments on the severity of disease progression as evident by (B) the change in body weight, (C) arthritic score, and (D) arthritic index are presented here. There was a significant suppression of both arthritic index and score by Rg3 and indomethacin treatment between day 20-42. Data are expressed as mean ± S.D of n = 6 animals per group. ***p<0.0001, **p<0.01, *p<0.05, oοοp<0.0001, οο p<0.01, versus CIA + vehicle group, using student’s t-test.

4A.2.9. Radiology and histopathology

Figure 4A.14C depicts the histological evaluation of the tibio-femoral joints of the experimental rats with the signs of severe arthritis and inflammatory cell infiltration in the CII-immunized animals. In the Rg3-treated groups, the degree of arthritis induced joint damage was significantly reduced. The synovial linings of the joints were smooth and normal, with slight indications of synovial hyperplasia or other characteristics of
inflammation (Figure 4A.13C and D). Indomethacin treated group also revealed pronounced protection against cartilage and bone erosion. It was apparent that Rg3 controlled the synovial cell infiltration.

The results of radiographic images taken after the formalin fixation of hind limbs on day 42 were corroborating with histopathological findings. The hind paws of vehicle-treated rats revealed bone matrix resorption in the tibiotarsal joint. Rg3 treatment markedly protected against bone resorption and soft tissue swelling. A similar protective effect in terms of reduced degree of arthritis severity was also observed in the group of animals treated with indomethacin (Fig 4A.13 C and D). There was no evidence of pathology in the sham group of rats (Fig 4A.13A). Through both histologic and radiographic evaluations, we have found that Rg3 at both the doses has considerable protective effects on the cartilage and bone in the immunized rats.

![Radiographic images](image.png)

![Histologic images](image.png)

**Figure 4A.14:** Effect of Rg3 treatment on arthritis progression as observed by anatomical pathology: (A)
representative radiographs of the hind limbs (showing the tibiotarsal and tibiofemoral joints), (a) control, (b) CIA+vehicle, (c) CIA+Rg3 (2 mg/kg body weight), d) CIA+Rg3 (20 mg/kg body weight), and (e) CIA+indomethacin. Arrows indicate the bone damage and space narrowing in both the joints. The encircled areas grossly showed the severe pathological conditions (swollen joints, edema) of the hind limbs. (B) Radiographic score, (C) representative histological figures (hematoxylin and eosin stained slides) of joints showing (a) smooth and monolayered synovial linings and uniform synovial space of control rats, (b) hyperplastic synovial cells, erosion and disruption of synovial linings was observed in CIA (c) smoother synovial lining and the synovium was nearly monolayered on treatment with Rg3 (2mg/kg) and (d) Rg3 (20mg/kg); (e) the synovial lining layer is mildly hyperplastic and monolayered thin synovium in indomethacin treated rats. Histological photomicrographs are representative of histopathology of five animals (n=5) per experimental group, (Original magnification×10). (D) Histological score. Results are expressed as mean ± standard deviation of 5 animals per group (n=5). *p<0.05, **p<0.01, versus CIA+vehicle group, using student’s t-test.

4A.2.10. Plasma cytokine levels

Significant increase (p<0.0001) in the levels of proinflammatory cytokines, TNF-α, IL1β and IL6 were found in the plasma of vehicle-treated CII immunized as compared to the healthy control rats (Figure 4A.14). Consistent with the morphological parameters of the animals, the levels of these proteins were significantly suppressed in CIA rats treated with Rg3 at both the doses (p<0.0001) and indomethacin (p<0.0001).

4A.2.11. Levels of anti-collagen antibody

In CIA, the effect of Rg3 on the humoral immunologic component was assessed by measuring the anti-CII antibody titer. Plasma anti-CII antibody titer was significantly increased in the CIA rats as measured on day 42 (Figure 4A.14E). Rg3 showed no significant inhibition on the anti-CII antibody formation. Negligible anti-CII antibody titers were found in the serum of control rats.

4A.2.12. NO level of rat plasma

Figure 4A.13D shows the effects of both the concentrations used for Rg3 on the
production of NO in the plasma of the experimental rats. The nitrite production was significantly elevated in the CIA rats ($p<0.0001$) fed with vehicle only, as compared to the control rats. Administration of Rg3 significantly ($p<0.0001$) reduced the nitrite concentration of plasma of the immunized rats. Treatment with indomethacin also yielded similar results ($p<0.0001$).

**Figure 4A.15:** Effect of Rg3 treatment on the pro-inflammatory cytokine levels in the plasma of CIA rats. Concentrations were expressed in pg/ml of (A) TNF-α, (B) IL1-β and (C) IL-6. (D) Efficacy of Rg3 and indomethacin treatment on the production of NO as measured by Griess-nitrite assay using NaNO$_2$ as standard. NO concentration was measured in both plasma and joint exudates. (E) Titers of the anti-type II collagen antibody treated and untreated CIA and healthy control animals. Plasma obtained from the blood obtained on day 42 from the rats treated with either vehicle or Rg3 (for 2 mg/kg, and 20 mg/kg). Results are presented as mean and SD of 5 animals per group, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$, $^\ddag p<0.05$, $^\ddag\ddag p<0.01$, $^\ddag\ddag\ddag p<0.0001$, versus CIA+vehicle group, using student’s t-test.

**4A.2.13. Effect of Rg3 on iNOS expression**

The onset of CIA significantly increased the expression levels of iNOS in comparison to the control rats. Consistent with the ELISA results, quantitative RT-PCR showed that the levels of iNOS were down-regulated by Rg3 treatment in a
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countent-dependent manner (Figure 4A.16). The inhibitive effect of Rg3 (20 mg/kg body weight) was a bit stronger than indomethacin. Both the doses of Rg3 significantly inhibited the expression of iNOS.

![Figure 4A.16: Effect of the active compound Rg3 on the gene expression of iNOS in Wistar rats. (A) Agarose gel showing the iNOS gene expression in the PBMCs. (B) Densitometric analysis of the bands as obtained for iNOS gene expression, G3PDH was used as housekeeping gene control. **p<0.01, ***p<0.001, oo p<0.001, ooop<0.0001, versus CIA+vehicle group, using student’s t-test.]


The CIA rats administered with vehicle only showed very less locomotor activity (p<0.001) in the arena compared to the control ones (Table 4A.2). Rg3-treated groups and indomethacin treatment significantly improved the locomotor and exploratory behavior of rats when compared with CIA (p<0.001).
Table 4A.2: Behavioral test of all the experimental rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CIA + vehicle</th>
<th>CIA + Rg3 (2mg/kg)</th>
<th>CIA + Rg3 (20mg/kg)</th>
<th>CIA + Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lines crossed</td>
<td>119.5 ± 39.5</td>
<td>8 ± 3.6</td>
<td>53 ± 15.5</td>
<td>81.5 ± 12.5</td>
<td>69.3 ± 9.7</td>
</tr>
<tr>
<td>Rearing</td>
<td>17.75 ± 7.13</td>
<td>0.6 ± 0.9</td>
<td>4.75 ± 2.75</td>
<td>9.5 ± 3.5</td>
<td>10 ± 3.6</td>
</tr>
<tr>
<td>Defecation</td>
<td>2 ± 1.05</td>
<td>5.53 ± 2.03</td>
<td>3.8 ± 1.4</td>
<td>3.16 ± 1.1</td>
<td>3.88 ± 2.16</td>
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</tbody>
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Table legend: Efficacy of Rg3 and indomethacin treatment on the motility assessed by the open field test and expressed by the mean of the number of squares covered by the rats of each group. Rg3 treatment significantly increased the motility of the arthritic animals in a dose dependent manner. Effect of Rg3 and indomethacin treatment on the load bearing capacity of hind limbs of rats as expressed by the mean of the number of rearing by the rats of each group. Rg3 treatment significantly increased the number of rearings of the arthritic animals in a dose dependent manner. Each value represents the mean ± S.D of n=5 animals per group. ***p < 0.001, *p < 0.05, ooo*<0.0001, versus CIA + vehicle group.

4A.3. Discussion

Plant kingdom is a rich source of active components that lead to the discovery and development of numerous agents that can be used as medicine against several diseases (Newman et al., 2000). Despite the recognized effects of several traditionally used medicinal herbs, their pharmacological activities have not been thoroughly investigated regarding their immunological effects. In this study, we have screened and selected whole plant methanolic extracts of some medicinal plants and examined their effects on the production of LPS-induced inflammatory mediators in J774 murine macrophage cell line. Initial screening of plant extracts was performed on the basis of their ability to inhibit the LPS-induced NO production by the macrophage. The cytotoxicity of all the plant extracts was determined on macrophage cells by MTT assay, and four active extracts that did not affect the cell viability up to a concentration of 500µg/ml were selected for further studies (results not shown). A variety of stimuli,
such as with LPS, TNF-α, and IFN-gamma can result in the production of massive amount of NO by the activated macrophages which can participate in the pathological processes in several acute and chronic inflammatory disorders (Kilbourn and Belloni, 1990; Murakami et al., 2003; Nathan, 1992). Therefore, drugs that decrease NO production by transcriptional down-regulation of iNOS gene expression, inhibiting the iNOS gene, major receptors for signaling initiated by LPS, depletion of arginine substrate by arginase, and/or enzyme activity, have appreciable therapeutic effect in the treatment of all major inflammatory and infectious diseases including some neurological diseases (Albina and Reichner, 1998; Boucher et al., 1999; Zhang and Ghosh, 2000). In this study, we demonstrate that the plant extracts under study significantly inhibited NO production (Figure 4A.1) in LPS-stimulated macrophages. Moreover, the inhibitory activity of these extract on LPS-induced NO production was found to be associated with the suppression of iNOS protein expression (Figure 4A.2).

Similarly, COX-2 is an inducible enzyme that catalyzes the production of prostaglandins, which contribute to the inflammatory process and tissue damage. The induction of prostaglandin production by LPS in macrophages is primarily due to the transcriptional activation of the COX-2 gene (Lee et al., 1992; Reddy and Herschman, 1994). It is reported that COX-2 can also be activated by high concentrations of nitric oxide, contributing towards more intense inflammatory responses as seen in many chronic inflammatory disorders (Hughes et al., 1999). Several natural products of plant origin have been shown to transmit their anti-inflammatory activities through suppression of COX-2 (Jachak, 2006), however, for that suppression of nitric oxide production is critical. It was observed that all the plant extracts were able to suppress the COX-2 protein expression in addition to iNOS in LPS-stimulated J774 macrophage cells (Figure 4A.2). The production of TNF-α and pro-inflammatory interleukins such as IL-1β and IL-12 is a crucial part of the immune response to many inflammatory stimuli.
For instance, overproduction of these mediators could be detected in both acute (septic and hemorrhagic shock) (Jachak, 2006), as well as chronic (rheumatoid arthritis, atherosclerosis) inflammatory disorders (Esposito and Giugliano, 2004b; Krakauer, 2004c; Ohshima and Bartsch, 1994b). Recently, new approaches on the use of herbal products for the treatment of inflammatory diseases by inhibiting inflammatory cytokines such as TNF-α and interleukins (Kim et al., 2005) has become an essential area of investigation because of their associated complications. Our results revealed that each of the plant extract could remarkably suppress the production of more than one inflammatory cytokine from LPS-activated macrophages (Figure 4A.3). In monocytes/macrophages engagement of toll like receptor-4 (TLR-4) with LPS or other microbial products is known to trigger several intracellular signal transduction cascades. Among the most prominent and best characterized of these is the activation of pro-inflammatory IκB kinase (IKK)-NF-κB pathway leading to NF-κB activation (Anderson, 2000; Li and Verma, 2002a). TLR-induced NF-κB activation represents a critical component of innate host defense system, which is phylogenetically conserved (Ghosh et al., 1998). This signaling pathway induces many genes that encode inflammatory mediators through the activation of transcription factor NF-κB (Baldwin, Jr., 1996). Therefore, these signaling molecules may represent novel targets for the treatment of patients with inflammatory diseases (Guha and Mackman, 2001). The most probable mechanism by which these plant extracts inhibit the expression of these proinflammatory mediators seems to involve the inhibition of NF-κB activation. It is evident from our results (Figures 4A.4, 4A.5) and it was verified that the anti-inflammatory effects caused by these plant extracts are through blockade of NFκB nuclear translocation and thus, the inhibition of LPS-stimulated expressions of the iNOS and COX-2 genes and production of cytokines. In addition to NF-κB activation, TLRs can also initiate mitogen-activated protein kinase (MAPK) signaling cascades that
induce phosphorylation of p38, ERK1/2, and c-Jun NH2-terminal kinase (JNK) and thus activation of several other transcription factors, including activator protein 1 (AP-1) and Elk-1 (Anderson, 2000; Beutler, 2000; Yang et al., 2000). However, the direct role of these transcription factors is not well established in transcriptional activation of pro-inflammatory genes in response to LPS stimulation but the possibility of inhibition of transcription factor(s) other than NF-κB by these plant extracts cannot be ruled out.

Using the rat CIA model and 2DE technique, a qualitative and quantitative analysis of acute phase plasma proteins (glycoproteins) was done. Immunization of genetically susceptible strains of rodents and primates with type II collagen leads to the development of a severe polyarticular arthritis that is mediated by an autoimmune response. Synovitis, erosions of cartilage and bones are hallmarks of CIA as in the case of RA. A wealth of evidence indicates that synovitis is initiated by the production of pathogenic autoreactive antibodies capable of fixing and activating complement1. The present study aims at the development of arthritic model and its evaluation by qualitative and quantitative analysis of markers. A major group of glycoproteins are the acute phase proteins. The concentration and glycosylation pattern of these proteins are altered in various pathological conditions. Here, we have considered two such acute phase proteins, CRP and haptoglobin for evaluation of successful arthritic model development.

Haptoglobin is heterotetramer consisting of 2a and 2b subunits joined by interchain disulfide bonds (Turner, 1995). There are 4 distinct asparagine residues (Asn 23, 46, 50, 80) in each β-chain and they display oligosaccharide heterogeneity. Fucosylated haptoglobin was also found to be increased in case of pancreas cancer and various other disease (Okuyama et al., 2006). The human CRPs purified from several different pathological samples were also found to be glycosylated (Das et al., 2003c) and is not associated with lipoproteins in blood plasma. The glycosylation pattern of CRP showed
the presence of sialic acid, glucose, galactose and mannose (Das et al., 2003b). C-reactive protein is produced by the liver following tissue injury and is thought to be an important component of the innate immune system implicating its critical role in the host defense mechanism. In spite of in vitro demonstration of the involvement of CRP in multiple functions, namely, activation of the complement system, opsonization, phagocytosis, binding to platelets, chromatin, histones and small nuclear ribonucleoprotein particles (Shrive et al., 1996; Volanakis, 2001), its precise physiological role is yet to be established. Till date human CRP levels are used only as a non-specific diagnostic marker. These observations may pave the way for overcoming some obstacles for the clinical use of CRP and haptoglobin.

Earlier the up regulation of acute phase protein such as CRP and haptoglobin was observed in RA patient as well as in other inflammatory disease. Plasma levels of CRP increases quickly during acute inflammation or infection suggesting a better indicator of disease activity. Normally there is negligible amount of CRP in normal blood serum. Although 1 mg/dL is usually considered high for CRP, most infections and inflammations result in CRP levels above 10 mg/dL.” (www.lifediagnosics.com). It is rapidly synthesized in liver in response to inflammatory cytokines and the serum CRP level increases to 1000-fold from the basal levels (0.1–0.5 μg/ml) within few hours(Das et al., 2003a). CRP is extensively used for monitoring disease activity in RA but haptoglobin response to stress is not as high as that of CRP hence providing weak indicator to severe disease activity. CRP and haptoglobin were found to show significant correlation with composite disease activity index, DAS (disease activity score) and confirmed the relevance of CRP in measuring disease activity in RA. Thus CRP is probably the single most useful molecule for monitoring acute phase reactions. However, the way in which CRP mediates in the acute phase reactions is still remains unclear.
The anti-arthritic effect of Rg3 was evaluated in rat CIA model. Coumarins are the important compounds present in this plant and are known to help it to adapt to abiotic and/or biotic stresses. Coumarins are found ubiquitously in higher plants contributing to the defense against plant pathogens (Vialart et al., 2011). The coumarin derivatives and newly synthesized coumarins have been exploited due to their broad spectrum of pharmacological activities including anti-oxidant, anti-inflammatory, anti-coagulant, anti-cancer, anti-microbial effects, etc (Riveiro et al., 2010). In an earlier work of our laboratory, a coumarin derivative, Rg1 with IUPAC name of (3-(1',1'-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin) was isolated from R. graveolens demonstrated anti-inflammatory activities in LPS-stimulated macrophage cells (Raghav et al., 2007).

In the present study, the anti-arthritic activities of Rg3, a novel compound isolated from the same plant has been investigated using CIA rat model. CIA is the closest experimental model used to investigate the pathogenesis of RA and used for identification of potential therapeutic agents (Choi, 2007; Hitchon and El-Gabalawy, 2004a).

Our results demonstrate that Rg3 (i) reduces the progression of CIA at both pathological and clinical levels as evident through reduction in arthritic score, index and body weight loss, ii) reduces the infiltration of the joints with polymorphonuclear cells (histopathology), 3) exerts significant protective effects against cartilage destruction in the affected knee joint (radiography), iv) down-regulates the level of key pro-inflammatory cytokines such as TNF-α, IL1-β and IL-6 in plasma, and v) increased the motility and rearing activity of CIA rats as studied in an open field test. All of these findings support the view that Rg3 attenuates the degree of arthritis and joint injury caused by CII in the rat.

It was clearly observed that Rg3 effectively improved the arthritic symptoms. Paw swelling measurement (expressed as arthritis index), an important parameter in the
determination of degree of inflammation is also indicative of the therapeutic efficacy of a given compound. A significant reduction of the inflammation in the hind paws of the immunized rats, Figure 4A.13, was observed in the treatment groups of Rg3 (2 and 20 mg/kg) and indomethacin when compared to their untreated counterparts suggesting the anti-arthritic potential of Rg3 at both the doses. The CIA inhibition by Rg3 at the dosage of 20 mg/kg (83.6%) was found to be closer to that of indomethacin (87.9%). The absence of weight loss in the Rg3-treated rats as opposed to the untreated CIA groups showed the low toxicity and recuperative effects of the compound.

The roentgenograms point up to the considerable decrease in the severity of the clinical indices viz., soft tissue swelling, bone erosion and joint space narrowing which were brought about by treatments with Rg3. Reduction of cartilage damage, massive cell influx and other histopathological features were also attained by Rg3 (2 and 20 mg/kg) demonstrating significant protective effects of the joints. Thus radiographic and histopathological results corroborate each other.

The measurable levels of cytokines such as TNF-α, IL-1β and IL-6 are found locally in synovial tissue, the major site of inflammation in CIA as well as RA. These are then circulated systemically through the plasma and facilitate the local and systemic inflammatory response.

However, inflammation is mediated by a milieu of inflammatory agents such as cytokines, prostanoids, prostaglandins, nitric oxide (NO), other reactive oxygen and nitrogen species, which are also the major targets for the treatment of inflammatory disorders. NO free radicals are produced by nitric oxide synthase (NOS) enzyme when L-arginine is converted to L-citrulline. Cytokines, such as TNF-α, IL-1β, IFN-γ, stress and bacterial infections cause the inducible NOS (iNOS) to be overexpressed (Nathan and Xie, 1994b). There is a synergistic effect of IL-1β with NO in the processes of osteoclast formation and impairment of cartilage proteoglycan synthesis (Taskiran et al.,
1994) resulting in bone erosion and subsequent joint damage. So, in the endeavor to prevent the detrimental effects of such inflammatory disorders both IL-1β and nitric oxide are important molecular targets.

Another important effect of IL1 and TNF-α is their contribution to the expression of adhesion molecules, which in turn facilitates the migration of cells to the site of inflammation and the stimulation of a variety of responses in endothelial cells (Maini et al., 1995). We confirm here that the onset of CIA was accompanied with a substantial increase in the levels of TNF-α, IL-1β and IL-6 in the plasma. Interestingly, the administration of Rg3 dramatically reduced the levels of these three pro-inflammatory cytokines. It is therefore proposed that Rg3, being an important negative regulator of these pro-inflammatory cytokines related to RA might deactivate the inflammatory response of infiltrating and proliferating synovial cells. Hence, it is conceivable to suggest that part of the beneficial anti-inflammatory and cartilage/bone protective effects of Rg3 may be mediated through the inhibition of TNF-α and IL-β. However, Rg3 showed no inhibitory effect on the increased titers of anti-collagen antibody in the plasma of the arthritic rats suggesting that no immunosuppression is associated with its therapeutic efficacy. We also studied the effect of Rg3 on cell viability using J774 cell lines and the nitrite (data not shown) which puts forward the low cytotoxicity as well as NO inhibition, thus substantiate the findings of the animal experiments.

The behavioral responses of rats such as locomotor activity and exploratory behaviors (rearing index) in an open arena were also measured in the open field test. The arthritic rats treated with vehicle only showed very less locomotor activity in terms of the number of squares crossed along with a lower rearing index. The probable reason for such a behavior is the stressed condition of the former and the inability to stand erect suggesting the reduced weight bearing ability over the hind paws. Treatment of CII-immunized rats by Rg3 resulted in an increased locomotion and load bearing. Rg3
has been proved to be a promising natural compound with the potential for clinical usage. In this chapter, the plants selected after the screening process were found to have anti-inflammatory effects. The novelty of the work lies in the fact that Rg3 is a novel active compound isolated from the *Ruta graveolens* and has been used for its anti-arthritic activity in CIA rat model for the first time. Further clinical investigations are needful in establishing it as a bedside drug for rheumatoid arthritis. Also, we have found that there is an alteration in fucosylation of the proteins Haptoglobin and C-reactive protein in the arthritic condition as compared to the healthy animals, this was not previously reported.

**Additional figures:**

**Figure 4A.S.1.** 2D DIGE images of the joint exudates proteins isolated from (A) CIA group (Cy3 labeled), (B) Control group (Cy5 labeled). (C) Proteins from both CIA and control groups labeled with Cy2, and (D) overlay of (A), (B) and (C).
Chapter 4A: Screening of plants for their anti-inflammatory properties and evaluation of natural compound for its anti-arthritic potency in CIA

Figure 4A.1. S.2. Coomassie stained 2DE images of the joint exudates proteins isolated from (A) CIA + vehicle, (B) control (C) CIA + Rg3 (2 mg/kg) (D) CIA + Rg3 (20 mg/kg) and (E) CIA + indomethacin groups. The differentially expressed protein spots were numbered and marked with arrows and ovals.

Figure 4A.S.2. Comparative analysis of various doses of Rg3 administered in the healthy control, untreated arthritic and CIA rats treated with various concentrations of Rg3 (2, 5, 10 and 20 mg/kg bodyweight/day), and indomethacin (1mg/kg/day). S.1.A. Arthritic index, S.1.B. Arthritic score and, S.1.C. Change in body weight.
### Table 4A.S. Plants screened for anti-inflammatory effects.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of plant</th>
<th>Common name</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Adonis vernalis</td>
<td>Pheasant's Eye</td>
<td>Ranunculaceae</td>
</tr>
<tr>
<td>2.</td>
<td>Urtica arense</td>
<td>Bladderwort</td>
<td>Urticaceae</td>
</tr>
<tr>
<td>3.</td>
<td>Quassia amara</td>
<td>Quassia</td>
<td>Simaroubaceae</td>
</tr>
<tr>
<td>4.</td>
<td>Commifera mukul</td>
<td>Gum gugul</td>
<td>Burseraceae</td>
</tr>
<tr>
<td>5.</td>
<td>Allium sativum</td>
<td>Garlic</td>
<td>Liliaceae</td>
</tr>
<tr>
<td>6.</td>
<td>Gelsemium sempervirens</td>
<td>Carolina jasmine</td>
<td>Loganeaceae</td>
</tr>
<tr>
<td>7.</td>
<td>Arnica Montana</td>
<td>Arnica</td>
<td>Asteraceae</td>
</tr>
<tr>
<td>8.</td>
<td>Chimaphilla umbellata</td>
<td>Common princes pine</td>
<td>Pyrolaceae</td>
</tr>
<tr>
<td>9.</td>
<td>Viscum album</td>
<td>Mistletoe</td>
<td>Loranthaceae</td>
</tr>
<tr>
<td>10.</td>
<td>Erictuicyon glutinosum</td>
<td>Yerba santa</td>
<td>Hydrophyllaceae</td>
</tr>
<tr>
<td>11.</td>
<td>Ruta gravedoens</td>
<td>Garden rue</td>
<td>Rutaceae</td>
</tr>
<tr>
<td>12.</td>
<td>Gingiber officinale</td>
<td>Ginger</td>
<td>Gingiberaceae</td>
</tr>
<tr>
<td>13.</td>
<td>Aletris farinosa</td>
<td>Colic root, stargass</td>
<td>Liliaceae</td>
</tr>
<tr>
<td>14.</td>
<td>Gaultheria procumbens</td>
<td>Teaberry</td>
<td>Ericaceae</td>
</tr>
<tr>
<td>15.</td>
<td>Salix nigra</td>
<td>Black willow</td>
<td>Salicaceae</td>
</tr>
<tr>
<td>16.</td>
<td>Hypericum perforatum</td>
<td>St. johswort</td>
<td>Hypericaceae</td>
</tr>
<tr>
<td>17.</td>
<td>Colchicum autumnale</td>
<td>Meadow saffron</td>
<td>Liliaceae</td>
</tr>
<tr>
<td>18.</td>
<td>Rhiz toxicodendron</td>
<td>Poison ivy</td>
<td>Anacardaceae</td>
</tr>
<tr>
<td>19.</td>
<td>Polygalan senega</td>
<td>Snakeroot</td>
<td>Polygaceae</td>
</tr>
<tr>
<td>20.</td>
<td>Grindelia robusta</td>
<td>Gumplant</td>
<td>Asteraceae</td>
</tr>
</tbody>
</table>
Suramin, a potent antioxidant and anti-inflammatory agent, ameliorates CIA
Chapter 4B: Suramin, a potent antioxidant and anti-inflammatory agent, ameliorates CIA

4B.1 Introduction

Suramin, has been widely used to treat trypanosomiasis, onchocerciasis (Jennings et al., 2002; Schulz-Key et al., 1985) and is being evaluated as an anticancer drug (Takano et al., 1994). It is an agent with broad-spectrum biological effects, including the ability to inhibit the binding of growth factors to their receptors, glycosaminoglycan degradation, membrane-associated ion pumps, protein kinase C, glycolysis, and cell motility (Grazioli et al., 1992). Suramin is known to have anti-TNF-α activity (Alzani et al., 1993). TNF-α is a critical pleiotropic cytokine and its overproduction has been implicated in the pathogenesis of a number of inflammatory diseases including rheumatoid arthritis (RA) (Agrawal et al., 2005; Nash and Florin, 2005; Raghav et al., 2006a; Rahman et al., 2009; Tracey and Cerami, 1994). As a potential debilitating, chronic autoimmune disease, RA mainly affects joints, characterized by synovial hyperplasia, inflammatory cell recruitment, and progressive destruction of cartilage and bone. Although the etiology of RA is still unclear, the sequence of events in the disease pathogenesis and the end-stage effector mechanisms are well established (Alonzi et al., 1998; Seo et al., 2004). Processes like mononuclear cell infiltration, synthesis of an array of degradative enzymes and production of inflammatory mediators like TNF-α, interleukin (IL)-1β and IL-6 result in joint damage (Feldmann et al., 1996c). The use of TNF-α and IL-1β antagonists in RA patients have shown substantial efficacy, but entails high cost, hypersensitivity to medications and infections (Bernatsky et al., 2007). The therapeutic effects in rheumatic diseases might be achieved by antagonizing additional pro-inflammatory cytokines, including TNF-α. Suramin has been demonstrated to impart its anti-TNF-α activity by promoting the dissociation of the biologically active trimeric form of TNF-α into inactive subunits, thus inhibiting the binding of TNF-α to its cellular receptors (Alzani et al., 1993; Alzani et al., 1995; Mancini et al., 1999). Suramin is also known to block the activity of IL-6 as it interferes with binding to its cell surface receptors (Strassmann et al., 1993). It also has anti-inflammatory properties as it suppresses myocardial inflammation in experimental autoimmune myocarditis (EAM) (Shiono et al., 2002). The effects of suramin in
ameliorating arthritic conditions are not yet studied; assuming that suramin is a partial TNF-α blocker, the present study was undertaken. Collagen induced arthritis (CIA), a well established animal model of RA, is frequently used to test new therapeutics (Durie et al., 1994; Rosloniec et al., 2010). The CIA model shares many pathophysiological properties with human RA, such as severe inflammation, mononuclear cell infiltration and cartilage and bone destruction (Durie et al., 1994). The purpose of this study was to examine whether administration of suramin could exert a therapeutic effect on CIA in experimental animals.

**4B.2 Results**

**4B.2.1 In vitro antioxidant assays**

Suramin as well as the reference standard antioxidants curcumin and L-ascorbic acid showed a concentration dependent activity in all the antioxidant assays done. IC$_{50}$ values were derived by using the concentrations ranging from 25 to 500 µg/ml (25, 50, 100, 250 and 500 µg/ml) of each compound tested in all the experiments. The percent activity or inhibition for the antioxidant assays were considered for the highest concentration used (500 µg/ml).

**4B.2.1.1 DPPH free radical scavenging activity**

Suramin exhibited (Figure 4B.1a) an inhibition of DPPH activity ($p<0.0001$) with IC$_{50}$ value of 41.5 ± 1.8 µg/ml greater than L-ascorbic acid (17.8 ± 0.9 µg/ml) and curcumin (25.4 ± 0.5 µg/ml). However, at 25 µg/ml, suramin had higher activity than L-ascorbic acid. At a concentration of 50 µg/ml and 250 µg/ml, suramin showed equivalent effect as of curcumin and L-ascorbic acid; at 100 µg/ml to that of L-ascorbic acid. Percent inhibition ($p<0.0001$) on DPPH radicals were similar in L-ascorbic acid (97.3 ± 0.7%) and curcumin (94.5 ± 1.2 %) and much higher than suramin (79.6 ± 0.8%).

**4B.2.1.2 Superoxide radical scavenging activity**
Superoxide radical scavenging activity of suramin was remarkably higher than both L-ascorbic acid and curcumin (Figure 4B.1b) in the concentration range of 25-500 µg/ml. The IC₅₀ values (p<0.0001) of suramin was 11.4 ± 0.6 µg/ml as compared to 330.5 ± 4.2 µg/ml and 398.5 ± 7.8 µg/ml for L-currumin and L-ascorbic acid, respectively. The percent superoxide radical scavenging activity (p<0.0001) of suramin (93.7 ± 1.5%) was much higher than that of curcumin (59.7 ± 0.6%) and L-ascorbic acid (56.2 ± 1.4%).

4B.2.1.3 Nitric oxide radical scavenging activity

Suramin exhibited a moderate dose-dependent nitric oxide scavenging activity (Figure 4B.1c) with an IC₅₀ value of 387.6 ± 8.6 µg/ml (p<0.0001) compared to L-ascorbic acid (408.8 ± 29.2 µg/ml) and curcumin (41.1 ± 2.2 µg/ml). At 250 and 500 µg/ml, suramin showed a bit higher activity than L-ascorbic acid. The potency in terms of % inhibition (p<0.0001) was highest for curcumin (75.6 ± 5.06%) followed by suramin (56.4 ± 3.5%) and L-ascorbic acid (53.4 ± 7.9%).

4B.2.1.4 Hypochlorous acid scavenging activity

Suramin had highest hypochlorous acid scavenging activity at 25 to 100 µg/ml concentrations. At 250 µg/ml, curcumin had equaled suramin and at 500 µg/ml the previous one showed highest effect. Suramin had a constantly higher activity than L-ascorbic acid, moreover at 25 and 50 µg/ml; the former showed almost double scavenging activity than the latter.

The results (Figure 4B.1d) showed the dose dependent hypochlorous acid scavenging activity of suramin (IC₅₀ = 87.4 ± 2.04 µg/ml) compared to that of reference antioxidants L-ascorbic acid (IC₅₀ = 176.2 ± 3.9 µg/ml) and curcumin (IC₅₀ = 106.3 ± 1.9 µg/ml) suggesting that suramin scavenged hypochlorous acid more efficiently than L-ascorbic acid. The % inhibition of hypochlorous acid by both suramin (81.048 ± 0.487%) and curcumin (85.6 ± 1.6%) are more than that of L-ascorbic acid (76.9 ± 0.2%).
4B.2.1.5 Hydrogen peroxide radical scavenging activity

Suramin scavenged hydrogen peroxide, proportionately with increasing concentration (Figure 4B.1e) and showed almost similar activity with that of curcumin at all concentrations but 100 µg/ml. When compared, the IC₅₀ values (p<0.01), hydrogen peroxide scavenging activities of suramin (237.7 ± 0.7µg/ml) and that of curcumin (196.2 ± 1.7 µg/ml) were almost equal. However, L-ascorbic acid (IC₅₀= 31.9 ± 0.9µg/ml) showed consistently highest activity at all the concentrations. The percent hydrogen peroxide scavenged (p<0.01, R²=0.74) at 500µg/ml, by L-ascorbic acid was highest (94.3 ± 0.3%) as compared to suramin (87.1 ± 0.6%) and curcumin (84.3 ± 0.4%).

4B.2.1.6 Total antioxidant capacity

Total antioxidant capacity of L-ascorbic acid and curcumin were observed to be much higher than that of suramin at 25 to 100 µg/ml but at 250 µg/ml all the compounds showed equal effects, however at 500 µg/ml both curcumin and L-ascorbic acid showed higher capacity than suramin (Figure 4B.1f). This assay exhibited the following sequence of total antioxidant capacity (p<0.0001, R²=0.8) in terms of optical density: L-ascorbic acid (2.05 ± 0.04) > curcumin (1.7 ± 0.04) > suramin (1.03 ± 0.05) 0.35 mg L-ascorbic acid equivalents per mg suramin and 0.25 mg gallic acid equivalents per mg suramin.
Figure 4B.1: Five different concentrations (25, 50, 100, 250 and 500μg/ml) of suramin and two known antioxidants curcumin and L-ascorbic acid were used in these assays. Inhibition of (A) DPPH radical formation, (B) superoxide, (C) nitric oxide, (D) hypochlorous acid, (E) hydrogen peroxide, radicals was measured. Total antioxidant capacity was presented in (F). All the assays were performed in triplicate and results were expressed as mean ± standard deviation (SD).

4B.2.2 In vivo anti-inflammatory effects of suramin

The effect of suramin on CIA rat model was studied for its anti-inflammatory activity.

4B.2.2.1 Clinical analysis in swelling of hind paws in CIA rats and effect of suramin
Chapter 4B: Suramin, a potent antioxidant and anti-inflammatory agent, ameliorates CIA

All the animals immunized with collagen developed severe inflammation. Hind limbs became severely red and edematous within 12–16 day period (Figure 4B.2a, paw photos). Inflammation or arthritic score (Figure 4B.2b) reached the highest of 8±1, 8±1.7 and 7±1.64 in the CIA animals treated with vehicle, suramin and indomethacin groups, respectively. Weight bearing on the affected limbs of vehicle-treated rats was poorly tolerated and swelling persisted 3–4 weeks, gradually culminated in a deformed joint. In both suramin and indomethacin treated animals, edema and redness began to subside gradually and showed a significant reduction (p<0.0001) of 63.3% and 70% in arthritic score, respectively, as compared to the ‘CIA+vehicle’ group after the treatment of 20 days. Severity of CIA as measured by arthritic index (Figure 4B.2c) showed a reduction of 76.2% in suramin and 83.4% in indomethacin treated rats. The CIA inhibition response by suramin treatment was 85.8% and that of indomethacin is 93.7% on day forty. Although the suramin-treated animals exhibited much lower AI, but the hind paw volumes did not return to fully normal condition.

4B.2.2.2 Effects of suramin on biological parameters

There was a drastic reduction of body weights (Figure 4B.2d) of the animals after the incidence of CIA (uniformly considered on day12) till the day of euthanization. However, suramin and indomethacin groups showed in significant weight gain of 15% and 39% relative to that of healthy control group, as opposed to CIA group which didn’t show any weight gain.
Chapter 4B: Suramin, a potent antioxidant and anti-inflammatory agent, ameliorates CIA

Figure 4B.2. Effect of suramin treatment on the progression of CIA. (A) Representative photographs of rat hind paws: (a) control, (b) CIA+vehicle, (c) CIA+Suramin, (d) CIA+indomethacin. (B) Mean arthritic score, (C) mean arthritic index, (D) Percent change in bodyweight with reference to day 12 (appearance of inflammation) till the day of euthanization (day 42). Results were expressed as mean±SD (n=5). ***p<0.0001, **p<0.01, *p<0.05, ○○○p<0.0001, ○○p<0.01, versus CIA+vehicle group, using student’s t-test.

4B.2.2.3 Radiographic and histopathological evaluation of suramin treatment on the joint damage in CIA rats

Typical radiographs of the hind-limbs in each group as shown in Figure 4B.3A were obtained on day 42. Radiographic joint damage including bone erosion and loss of joint space was detected in X-rays of paws of rats from CIA group (Figure 4B.3Ab), unlike paws of control rats (Figure 4B.3Aa). Compared with arthritic rats treated with vehicle, the suramin dosage reduced bone erosion changes and maintained joint space in arthritic rats.
treated on day 21 and onwards. There is a significant inhibition of joint damage and bone loss by the treatment of suramin.

Hematoxylin and eosin stained knee joint tissue slides were analyzed for histological evaluation and representative photomicrographs are given in Figure 4B.3B. Active synovitis and bone erosion were detected in CIA (b) but not in the control (a). Arthritic joints in CIA showed synovial hyperplasia, pannus formation, massive inflammatory cell infiltration (monomorphonuclear and polymorphonuclear cells), erosion of bone and cartilage. No such arthritic features of inflammation and tissue destruction were evident in the joints of healthy control groups. Both suramin and indomethacin treatment significantly reduced the severity accompanied by reduction of cartilage damage, massive cell influx.
Figure 4B.3: (A) Representative radiographs of the hind limbs (showing the tibiotarsal and tibiofemoral joints) of rats from all experimental groups, (a) control, (b) CIA + vehicle, (c) CIA + suramin and (d) CIA + indomethacin. Arrows indicate the bone damage and space narrowing in both tibiotarsal as well as tibiofemoral joints. The encircled areas grossly show the severe pathological conditions of the hind limbs, such as swollen joints and edema. (B) Representative histological figures (hematoxylin and eosin stained slides) of knee joints from all experimental groups of rats showing (a) smooth and monolayer synovial linings and uniform synovial space of ‘control’ rats, (b) hyperplastic synovial cells, erosion and disruption of synovial linings was observed in ‘CIA + vehicle’ (c) smoother synovial lining and the synovium was nearly monolayered on treatment with suramin and (d) the synovial lining layer is mildly hyperplastic and monolayer or double layer thin synovium was evident in indomethacin treated rats. Histological photomicrographs are representative of histopathology of five animals (n=5) per experimental group. (Original magnification ×10).

4B.2.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

Pathogenesis of RA includes the secretion of high levels of pro-inflammatory cytokines by macrophages and other cells. TNF-α, IL-1β and IL-6 contribute to systemic inflammatory conditions and joint destruction. Incidentally, in the present study all the three cytokines showed significantly higher levels in CIA rats. These levels were drastically reduced after suramin and indomethacin treatment (Fig. 4B.4). Plasma cytokines quantified of all the three groups showed 29.4% reduction in TNF-α by the treatment of suramin (*p<0.05). Similarly, there were significant reductions (p<0.0001) in IL1β and IL6 concentrations, respectively, as a result of suramin treatment. However, the level of IL-6 in plasma was not reduced significantly in the indomethacin-treated animal.
Figure 4B.4: Cytokine levels were measured in both plasma and joint homogenates and concentrations were expressed in pg/ml of (A) Tumor necrosis factor-α (TNF-α), (B) Interleukin-1β (IL1-β) and (C) Interleukin-6 (IL-6).

4B.2.3 Acute phase plasma proteins

WGA affinity chromatography helped to enrich plasma glycoproteins and deplete highly abundant proteins such as albumin. The 2DE profiling of WGA bound plasma proteins revealed the presence of multiple protein spots, which were differentially expressed in the control and CIA rats (Fig. 4B.5A, B). The protein spots which were brought to normal levels after suramin treatment were identified by MALDI-TOF (Table 4B.1). The results made it apparent that most of the normalized spots in the two dimensional gel were acute phase proteins (APPs), such as orosomucoid 1 precursor, hemopexin and alpha-1 major acute phase protein (T kininogen-1). The expression of T kininogen-1 was found to be very high in CIA rats, which was further validated by Western blot analysis (Fig. 4B).

Fig. 4B.5. (A) 2DE profile of WGA bound plasma glycoproteins. (B) Validation of kininogen (Spot no. 6) by 2DE Western blotting.
Table 4B.1: List of differentially expressed plasma glycoproteins identified by MALDI-TOF MS MS. Upregulated (↑) or downregulated (↓) glycoproteins in CIA rats treated with vehicle.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Accession no.</th>
<th>*M.W./P.I</th>
<th>Precursor ions</th>
<th>Ion score</th>
<th>Relative ¥ difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Clusterin</td>
<td>gi</td>
<td>461756</td>
<td>51,375/5.4</td>
<td>1800.9403</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Orosomucoid 1 precursor</td>
<td>gi</td>
<td>16757980</td>
<td>23,731/5.6</td>
<td>1884.8402</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>Hemopexin</td>
<td>gi</td>
<td>1881768</td>
<td>51,350/7.5</td>
<td>1211.7320</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>Corticosteroid-binding globulin precursor</td>
<td>gi</td>
<td>57527135</td>
<td>44,813/4.8</td>
<td>1544.8136,1824.9833</td>
<td>77,104</td>
</tr>
<tr>
<td>5</td>
<td>LMW K-kininogen precursor</td>
<td>gi</td>
<td>205077</td>
<td>36,471/5.9</td>
<td>1009.5463,3463.3181</td>
<td>39,143</td>
</tr>
<tr>
<td>6</td>
<td>Alpha-1 major acute phase protein (T-kininogen-1)</td>
<td>gi</td>
<td>205308</td>
<td>47,755/6.0</td>
<td>1815.8030</td>
<td>112</td>
</tr>
<tr>
<td>7</td>
<td>Hemopexin</td>
<td>gi</td>
<td>1881768</td>
<td>51,350/7.5</td>
<td>1726.7223</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>Beta-2-glycoprotein</td>
<td>gi</td>
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<td>1529.7823,2365.0318</td>
<td>66,40</td>
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<tr>
<td>9</td>
<td>Hemopexin</td>
<td>gi</td>
<td>1881768</td>
<td>51,350/7.5</td>
<td>2472.0557</td>
<td>75</td>
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<tr>
<td>10</td>
<td>Vitronectin</td>
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<td>1255044</td>
<td>55,364/5.6</td>
<td>2000.0673,2146.0005</td>
<td>154,121</td>
</tr>
</tbody>
</table>

* Molecular weight (M.W) and isoelectric point (P.I) as available in SwissProt data base.

¥ Relative percent difference in comparison to control animals

4B.2.4 **In vivo** antioxidant effects of suramin
4B.2.4.1 Reduced glutathione

The reduced glutathione levels measured as acid soluble sulfhydryl group (-SH) in joints, liver, spleen and kidneys of experimental and control animals are shown in Figure 4B.6A. GSH level was found to be significantly increased in CII immunized rats as compared to the control ones. The administration of suramin significantly reduced the enzymic antioxidant level in arthritic rats to basal limits.

4B.2.4.2 Lipid peroxidation and protein oxidation

The changes in the levels of TBA-RS and protein carbonyl content as an index of lipid peroxidation and protein oxidation respectively were measured in the plasma and cytosolic fraction of joints, spleen, liver and kidneys of all the experimental animals and the obtained results were presented in Figure 4B.6B. The highest LPO was observed in the spleen followed by kidneys, joints, plasma and liver of arthritic rats when compared to the control. Plasma from arthritic rats showed highest protein carbonyl content (Figure 4B.6C) followed by kidney, joints and spleen and the lowest activity was seen in the liver when compared with the control. Synchronous administration of suramin to arthritic rats altered the above changes to nearly that of the normal levels by significantly inhibiting the accumulation of MDA and protein carbonyl content in plasma, joints, liver, kidney and spleen of arthritic rats. Intraperitoneal treatments of arthritic rats with indomethacin have effects similar to those of suramin treatment.

4B.2.4.3 Tissues along with plasma antioxidant enzyme status

The antioxidant enzymes SOD, catalase, GST, GPx and GR activities were determined in the joints, spleen, liver and kidney along with plasma of arthritic and treated rats and compared with age-matched control groups. In the arthritic rats, the highest activity of SOD, catalase, GST and GPx were found in the spleen, the highest activity of GR was found in the joints followed by spleen and the lowest activity of GR and GST was seen in the liver. In contrast, the second highest activity of SOD, catalase and GPx was observed in the liver
being a good contributor of biological antioxidant defense system. In arthritic rat plasma, the levels of GR, catalase, GST, GPx and SOD, increased by 2.7, 2.5, 2.4, 1.5 and 1.4 fold, respectively, when compared to control rats. The arthritic joints showed an increase of 7.03 (GR), 2.4 (Catalase), 1.9 (GST) and 1.6 (SOD) in comparison with the lowest activity of GPx among all other organs. Treatment with suramin normalized the altered antioxidant enzyme levels of all tissues affected due to arthritis towards their control values. Indomethacin treatment also showed similar results.
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Fig. 4B.6: The *in vivo* antioxidative potential of suramin (10mg/kg/day) and indomethacin (1mg/kg/day) evaluated by antioxidant assays using the plasma of rats from each group and the tissue homogenates of the dissected organs (liver, spleen, kidneys and joints) with different antioxidant tests: (A) GSH results were expressed as μM GSH/g tissue (B) LPO in nmoles of MDA/mg protein (C) Protein oxidation in nmoles of carbonyl content/mg protein (D-H) SOD, CAT, GST, GPx and GR in units/mg protein.
4B.3. Discussion

In continuation of our earlier studies on anti-arthritic compounds from medicinal plants (Raghav et al., 2006b; Raghav et al., 2007), the present work was aimed at investigating anti-arthritic potential of suramin using well established CIA model of polyarthritis. The anti-arthritic activity of suramin was comparable to a first line drug, indomethacin (Hassan et al., 1991; Sengodan et al., 2009), even though suramin could not completely reverse the damage due to arthritis.

The results from in vitro studies showed the antioxidative potential of suramin, which was comparable with standard antioxidants, curcumin and L-ascorbic acid. The inhibitory effects of suramin on superoxide, hydrogen peroxide, hypochlorous acid, nitric oxide radicals and the total antioxidant capacity were determined. Suramin showed markedly higher superoxide anion inhibitory activity than both the standards whereas; it was found to be comparable with either or both of the standards in all other assays.

Studies for the anti-arthritic properties of suramin were carried out using CIA rats. In RA as well as CIA, the primary site of inflammation has been the synovial tissue, which releases cytokines into the systemic circulation causing increased plasma levels of TNF-α, IL-1β and IL-6. The circulating cytokines may alter the function of distant tissues, including adipose, skeletal muscle, liver and vascular endothelium. Spleen, an immune organ, is also affected in RA as evidenced by enlarged spleen 5–10% of the patients with RA (Hassan et al., 1991; Nishiya et al., 2000). Inhibition of the pro-inflammatory cytokines in plasma and joint exudates is expected to relieve arthritis associated discomforts. Suramin indeed exerted inhibitory effect against cytokines (Fig. 4.3) indicating its beneficial anti-inflammatory and cartilage/bone protective efficacy. The cytokine inhibition also led to reduced production of free radicals which further enhanced the protection (Bkaily and Orleans-Juste, 1999). Doses of 100 mg and 200 mg/kg body weight of suramin have been used earlier to ascertain the healing effects on colonic anastomoses (Hendriks et al., 1999). Our studies showed that a dose of 10 mg/kg/day of suramin had therapeutic efficacy (Fig.4.1D) similar to that obtained
with 50 or 100 mg/kg (data not shown), but the rats treated with higher doses demonstrated more weight loss and weakness. Other pharmacokinetic studies showed that suramin was not lethal up to an oral dose of 500 mg/kg body weight (Ogden et al., 2004). Hendriks et al. demonstrated that the maximal tolerable intraperitoneal dose of suramin for rats was 200 mg/kg with 100% survival (Hendriks et al., 1999). Based on antiarthritic potency determined by reduction in paw swelling, arthritic score and recovery from body weight loss in CIA rats, indomethacin (1 mg/kg body weight) showed better results (Fig.4.1). Indomethacin is a non-steroidal indole derivative which has been reported to be an effective anti-inflammatory agent appropriate for long term use in RA, ankylosing spondylitis and osteoarthritis (Hassan et al., 1991; Sengodan et al., 2009). However, toxicity of indomethacin has always been a limiting factor in its long term use. The oral LD50 of indomethacin in mouse was 11.8 mg/kg as compared to i.p LD50 750 mg/kg of suramin (Cayman chemicals and Santa Cruz Biotechnology datasheets). Suramin (known as Germanin and Bayer 205) has been used to treat trypanosomiasis and onchocerciasis (Jennings et al., 2002; Schulz-Key et al., 1985). It is also under clinical evaluation as a potent anticancer drug (Gradishar et al., 2000; Knox and Moore, 2001; Small et al., 2000; Song et al., 2004). The extremely diverse range of biologically important molecules and cell lines that suramin has been reported to inhibit is perhaps due to its non-specific mode of binding (McGeary et al., 2008). Suramin is reported to impair protein synthesis in vitro by inhibiting both initiation and elongation (Brigotti et al., 2006). It has been widely demonstrated that the progression of RA is reflected by the concentration of APPs in plasma and proteins which form a panel of disease biomarkers (Raghav et al., 2006c; Saroja et al., 2011; Saroja and Das, 2011). Therapeutic agents that affect the acute phase plasma protein profile in RA not only impart relief from the disease symptoms but can also ensure intervention in the disease progression itself. Therefore, we investigated the effect of suramin on the expression of plasma proteins in CIA rats. A WGA column having affinity for GlcNAc, chitooligosaccharides and sialic acid, was used to enrich glycoproteins before 2DE. WGA bound plasma protein profiling showed the
differential expression of many APPs like clusterin, orosomucoid 1 precursor, hemopexin and alpha-1 major acute phase protein (T-kininogen1) in CIA rats which were normalized after suramin treatment (Fig. 4). Orosomucoid1 (ORM-1) or alpha-1-acid glycoprotein (α1 AGP) was earlier reported to be a good marker of RA in human, (Saroha et al., 2011) was highly up regulated in CIA rat plasma. Down regulation of clusterin (Apolipoprotein J) in CIA rat was very similar as reported in systemic lupus erythematosus (SLE) (Newkirk et al., 1999). Hemopexin, a heme binding plasma glycoprotein, known to prevent bacteria induced inflammation, was also found to be increased in CIA rats, like other arthritis models (Liang et al., 2009). T-kininogen (alpha-1 major acute phase protein) a rat specific kinin, is known to be involved in various inflammatory responses where its expression is differentially regulated (Isordia-Salas et al., 2005). Similarly, in CIA rats we have found the over expression of high molecular weight T-kininogen-1. However, the reversal of positive acute phase proteins after suramin treatment further substantiated its anti-inflammatory activity.

There is wealth of evidence, both direct and indirect, that the production of free radicals at inflammatory sites damage cellular components in cartilage directly (Henrotin et al., 2003a). Reaction of NO with superoxide anion can modify the free and or/protein-bound amino acid residues and inhibit the enzyme activities. Their reaction also forms peroxynitrite, a potent oxidizing molecule capable of eliciting lipid peroxidation (Hitchon and El-Gabalawy, 2004b). In this study, we observed a significant increase in the lipid peroxide and protein carbonyl levels with the onset of arthritis. Peroxidation of lipids disturb the assembly of the membrane causing changes in fluidity and permeability, alterations of ion transport and probably contributes to accelerated atherosclerosis in RA (Hitchon and El-Gabalawy, 2004b). Oxidative modification of proteins leads to diminished specific protein functions and affect a variety of cellular functional proteins like receptors, signal transduction mechanisms, transport systems, and enzymes (Surh and Packer, 2005). Treatment with suramin decreased malondialdehyde and protein carbonyl concentrations in the joints, spleen and kidney. It can be thought that the beneficial effects of suramin
against accumulation of lipid and protein damage may be partly related to its direct free radical scavenging and antioxidative potential as discussed above. No significant difference (p>0.05) was observed with respect to protein oxidation in liver and plasma of arthritic rats. This in vivo lipid peroxidation inhibitory potential of suramin is well supported by our in vitro tests in which the concentration-dependent decrease in the MDA level signifies the potential of suramin in protecting against damage to membrane functions.

The concentration of GSH reflects the endogenous defenses against hydrogen peroxide formation and organic peroxides. Its reduced level in vehicle treated CIA rats might be due to its excessive consumption during oxidative stress and cellular lysis associated with the development of arthritis (Hassan et al., 2001). The reduction in the level of this non-enzymic antioxidant also contributes to increased lipid peroxidation in CIA rats as compared to healthy control rats (Kim et al., 2010). In group of rats treated with suramin and indomethacin, levels of GSH rescued to reach normal levels probably either by competing in scavenging for free radicals or by the direct detoxification of reactive oxygen species and/or neutralization of reactive intermediate species generated from oxidative stress.

A growing body of evidence indicates that superoxide radicals also result in collagen degradation that may accelerate other inflammatory reactions and tissue destruction through the recruitment and activation of neutrophils (Salvemini et al., 2001). SOD is a key antioxidant enzyme that protects cell from superoxide toxicity. Besides, SOD has a double-barreled impact because its degradation product that is, hydrogen peroxide interacts with glutathione in the presence of GPx and ultimately forms water and oxygen. To this end, CAT also protects the cells by dismutating hydrogen peroxide into water and oxygen or by using it as an oxidant. The activities of these three enzymes were found to be increased in the tissues and plasma of vehicle treated CIA rats. The highest activities of SOD and CAT in arthritic joints and spleen and that of GPx in spleen and liver make these tissues more susceptible to oxidative damage by the action of oxygen free radicals. Indeed increased
ROS/superoxide radicals have been documented at inflammation sites such as synovial joints, which in turn contribute to lipid peroxidation by increasing hydrogen peroxide levels. Thus, to respond to an increase in superoxide radicals, the cells must not only elevate SOD but CAT and GPx as well, the latter to counter the effect of hydrogen peroxide. Our literature survey revealed a very high discrepancy in the antioxidant system findings. As to SOD activity, significant decreases (Karatas et al., 2003); (Kiziltunc et al., 1998) and increases (Geetha et al., 1998a); (Kasama et al., 1988) were reported. Related to CAT and GPx defense system; similar to our data significantly high activities of enzymes were observed (Geetha et al., 1998b); (Kasama et al., 1988); (Braven et al., 1989) in RA patients. Another important enzyme of the glutathione defense system, i.e., GR regenerates reduced glutathione from oxidized glutathione at the expense of NADPH. As expected, the GR activity was also found to be increased in the tissues examined along with plasma (Chang et al., 1978). The highest augment of the activities was seen in joints and spleen of arthritic rats, which could be due to its induction to counter the effect of chronic oxidative stress that arises due to arthritis. Also, the increased GR activity is important for the detoxification pathway of oxygen free radicals. After suramin treatment, the alterations produced in the arthritic rats with respect to antioxidants concentrations were modulated to near normal levels. Therefore, reversal of free radical induced oxidative damage by suramin and indomethacin treatment indicates its protective nature against protein oxidation through glutathione redox cycle and ability to directly detoxify the ROS generated from exposure to CII.

GST is the detoxicant enzyme that metabolize toxic electrophiles, detoxifies endogenous compounds such as peroxidised lipids and is considered to be secondary antioxidant enzyme (Hayes and Pulford, 1995). In the present study, we have obtained marked increase in the activity of GST with the onset of CIA, in line with the findings of (Ostalowska et al., 2006) for RA. The increase in the activity of antioxidant enzyme can be attributed to response of the tissues to the increased oxidative stress due to CIA thereby suggesting an increased defense of the body against the effects of toxic electrophilic
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chemicals and hydrophobic compounds. This biochemical alteration was significantly ameliorated nearly to control values after administration of suramin and indomethacin to CIA rats. In the above work, suramin was checked for its anti-arthritic activity using many parameters such as anti-oxidant potential using various assays and this is the first report where the anti-oxidant potential of suramin has been explored. The comparative account of the plasma proteomic profiles of the healthy control and CIA rats showed that there have been differential expression (up-regulation and down-regulation) of glycoproteins which have been normalized with the treatment of suramin at the standardized dosage.
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Additional figure(s):

**Figure 4B.S1.** Images of silver stained 2DE gels of the joint exudates proteins isolated from (A) CIA + vehicle, (B) control, (C) CIA + Rg3 (2 mg/kg), (D) CIA + Rg3 (20 mg/kg) and (E) CIA + indomethacin groups. The differentially expressed protein spots were numbered and marked with arrows and ovals.

**Figure 4B.S2.** Comparative analysis of various doses of suramin administered in the healthy control, untreated arthritic and CIA rats treated with various concentrations of suramin (10, 50 and 100 mg/kg bodyweight/day), and indomethacin (1mg/kg/day). S.1.A. Arthritic index, S.1.B. Arthritic score and, S.1.C. Change in body weight.