CHAPTER 5

5.1 ANTIINFLAMMATORY AND ANTIARTHRITIC EVALUATION

5.1.1 Introduction

Natural products have been a major source of drugs from time immemorial. Many natural products have found their place for a variety of diseases. Herbal medicines have been used for the relief of pain throughout history (Almeida et al., 2001). The treatment of rheumatic disorder is an area in which the practioners of traditional medicine enjoy patronage and success (Akah and Nwambie, 1994). Inflammation is usually a protective response intended to eliminate the initial cause of cell injury. It is the part of the host defense mechanism which includes several tissue factors or mechanisms and also involves complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair (Vane and Botting, 1987). During the inflammation the release of histamine, bradykinin, 5HT, prostaglandins, leukotrienes, platelet activating factor (PAF), oxygen and nitrogen radicals play a pivotal role (Halliwell et al., 1988). It is known that anti-inflammatory action may be elicited by a variety of chemical agents and that there is no remarkable correlation between their pharmacological activities and chemical structure (Sertie et al., 1990). This fact associated with the complexity of the inflammatory process, makes the use of different experimental models essential when conducting pharmacological studies.

The acute inflammatory reaction is readily produced in animals with the help of irritant substances such as carrageenan, formalin, bradykinin,
histamine, 5HT, mustard and egg white when injected in the dorsum of the foot of the rats, they produce acute paw edema. Carrageenan induced paw edema model is the most commonly used in experimental pharmacology (Kulkarni, 1999). The subacute inflammation model is the cotton pellet granuloma method (Swingle and Shideman, 1972). In this study, a simultaneous assessment of the effects of the drug against the acute and proliferative inflammatory changes have been studied.

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease predominantly affecting the joints and periarticular tissue. RA still remains a formidable disease, being capable of producing severe crippling deformities and functional disabilities (Shin et al., 2003). RA is classified as an inflammatory arthritis, the disease comprises of three basic interrelated processes like inflammation, synovial proliferation and joint tissue destruction. The focus of RA is the synovial lining. RA factor containing immune complexes found in the joints activate the pathological process. Tumour necrosis factor alpha (TNF-α), is the product of macrophages have been demonstrated to play an important role in the pathogenesis of RA.

RA exerts powerful effects on the immune system, including induction of pro-inflammatory mediators such as interleukin 1, nitric oxide, prostaglandins, metalloproteases and adhesive molecules. The inflammation causes edema of the synovium and infiltration with mononuclear cells, macrophages, lymphocytes and plasma cells. The activated macrophages, lymphocytes and fibroblasts produce a variety of cytokines that promote further synovial proliferation and inflammation. Synovial fluid in RA contains various PG’s mainly PG E₂, leukotriene B₄, TNF alpha, interleukins and other cytokins.
It is now believed that monokines IL 1 and IL 6 and TNF alpha are the central mediators of active rheumatoid process (Satoskar et al., 2003).

Adjuvant induced arthritis in rats is an established model to study the physiological, biochemical and pharmacological aspects of arthritis. The chronic poly arthritis induced in rats is extensively used to study the mode of action of NSAIDs’s (Pearson, 1966, Swingle, 1974). Freund’s adjuvant induced arthritis has been used as a model of sub-chronic or chronic inflammation in rats and is of considerable relevance for the study of pathophysiological and pharmacological control of inflammatory processes, as well as the evaluation of analgesic potential or antiinflammatory effects of drugs (Butler et al., 1992; Besson and Guilbaud, 1988). One of the reasons for the wide utilization of this model is due to the strong correlation between the efficacy of therapeutic agents in this modal and rheumatoid arthritis in humans (Anderson et al., 2004).

Many species of mycobacteria (M.tuberculosis, M.butyricum, M.Phlei), Nocardia asteroides (Flax and Waksman, 1963) and Corynebacterium rubrum (Paronetto, 1970) are capable of inducing adjuvant – induced arthritis in rats. The development of adjuvant arthritis is thought to be T-cell mediated delayed – type hypersensitivity reaction, which is analogous to rheumatoid arthritis (Harris, 1981). After the induction of arthritis with Freund’s adjuvant the initial inflammatory response is developed within hours but more critical clinical signals emerge from the 10th post – inoculation day and thereafter, the alteration remain detectable for several weeks (Colpaert et al., 1982).

The present study extracts of the Ricinus communis root and Cassia fistula stem bark (RCA, RCM, CFA, CFM) were evaluated for their
anti-inflammatory (Carrageenan induced model and Cotton pellet granuloma model) and antiarthritic (Freund’s Adjuvant-Carageenan model) activities in experimental animals.

5.2 Materials and Methods

5.2.1 Animals

Wistar albino rats of either sex were used for the experimental study as given in the section 3.1.2.1

5.2.2 Anti-inflammatory evaluation

5.2.2.1 Carageenan induced paw edema model:

5.2.2.1.1 Experimental protocol

The following experimental protocol was used to assess the anti-inflammatory activity. The animals were divided into 10 groups of six animals each.

<table>
<thead>
<tr>
<th>Group</th>
<th>-</th>
<th>Control</th>
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<tbody>
<tr>
<td>Group I</td>
<td>-</td>
<td>RCA (250 mg/kg/po)</td>
</tr>
<tr>
<td>Group II</td>
<td>-</td>
<td>RCA (500 mg/kg/po)</td>
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<tr>
<td>Group III</td>
<td>-</td>
<td>RCM (250 mg/kg/po)</td>
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<td>Group IV</td>
<td>-</td>
<td>RCM (500 mg/kg/po)</td>
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<tr>
<td>Group V</td>
<td>-</td>
<td>CFA (250 mg/kg/po)</td>
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<tr>
<td>Group VI</td>
<td>-</td>
<td>CFA (500 mg/kg/po)</td>
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<tr>
<td>Group VII</td>
<td>-</td>
<td>CFM (250 mg/kg/po)</td>
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<tr>
<td>Group VIII</td>
<td>-</td>
<td>CFM (500 mg/kg/po)</td>
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<tr>
<td>Group IX</td>
<td>-</td>
<td>Reference standard Diclofenac sodium (5mg/kg/po)</td>
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<tr>
<td>Group X</td>
<td>-</td>
<td>Reference standard Diclofenac sodium (5mg/kg/po)</td>
</tr>
</tbody>
</table>
Procedure

Paw edema was induced by injecting 0.1ml of 1% carrageenan in physiological saline into the subplantar tissues of the left hind paw of each rat (Winter et al., 1962). The extracts (RCA, RCM, CFA,CFM) were administered orally 30 min prior to carrageenan administration. The paw volume was measured at intervals of 60, 120, 180, 240 minutes by the mercury displacement method using a plethysmograph. The percentage inhibition of paw volume in drug treated group was compared with the carrageenan control group (Group I). Diclofenac sodium (5 mg / kg / po ) was used as reference standard.

5.2.2.2  Cotton pellet granuloma model:

5.2.2.2.1 Experimental setup

The animals were divided into 6 groups of 6 each

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
</tr>
<tr>
<td>II</td>
<td>RCA (500 mg/kg/po)</td>
</tr>
<tr>
<td>III</td>
<td>RCM (500 mg/kg/po)</td>
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<tr>
<td>IV</td>
<td>CFA (500 mg/kg/po)</td>
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<tr>
<td>V</td>
<td>CFM (500 mg/kg/po)</td>
</tr>
<tr>
<td>VI</td>
<td>Reference standard Diclofenac sodium (5mg/kg/po)</td>
</tr>
</tbody>
</table>

Procedure

Wistar albino rats (170 -200 gm) of either sex were divided into 6 groups of 6 animals in each group. Cotton pellets weighing 20±1mg were autoclaved and implanted subcutaneously along flanks of axillae and groin
region of each rat (D’Arcy et al., 1960). Group I served as control and received the vehicle. The extracts (RCA, RCM, CFA, CFM), reference drug and vehicle were administered orally as per protocol to rats everyday for a period of 7 days. On the 8th day the animals were sacrificed by cervical decapitation and the cotton pellets were removed surgically, freed extraneous tissue, dried in an oven at 60°C, weighed and compared with control.

5.2.2.3 Anti-arthritic evaluation

5.2.2.3.1 Complete freund’s adjuvant – Carrageenan induced model

5.2.2.3.1.1 Experimental protocol

The animals were divided into seven groups of six animal each.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>I</td>
<td>Control</td>
</tr>
<tr>
<td>II</td>
<td>Negative Control</td>
</tr>
<tr>
<td>III</td>
<td>RCA (500 mg/kg/po)</td>
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<tr>
<td>IV</td>
<td>RCM (500 mg/kg/po)</td>
</tr>
<tr>
<td>V</td>
<td>CFA (500 mg/kg/po)</td>
</tr>
<tr>
<td>VI</td>
<td>CFM (500 mg/kg/po)</td>
</tr>
<tr>
<td>VII</td>
<td>Reference standard Diclofenac sodium (5 mg/kg/po)</td>
</tr>
</tbody>
</table>

Procedure

Arthritis was induced experimentally (Mizushima et al., 1972), in rats by complete freund’s adjuvant. 0.1 ml of complete freund’s adjuvant was inoculated intra-dermally at the base of tail to the all groups of animals except solvent control (group I). 10 days later all the animals were injected with 0.1 ml
of carrageenan (2% w/v in saline solution) into the subplantar aponeurosis of the right hind paw, except for solvent control.

Group II served as negative control. The other groups of animals treated with plant extracts (RCA, RCM, CFA, CFM), 1 hr before the carrageenan injection. Drug treatment started on 10th day and terminated on 21st day. The hind paw swelling was recorded from 10th day to 21st day. On 22nd day the animals were sacrificed by cervical decapitation and the blood was collected by retro orbital puncture prior to the sacrifice. The liver was rapidly removed and washed with ice-cold saline. 10% homogenate was prepared by using Tris-buffer (0.01 M, pH 7.4).

The haemolysate was extracted as per the method of Quist (1980). The blood collected with anticoagulant was centrifuged to remove the plasma. The packed cells were washed with isotonic saline to remove theuffy coat and then thrice with isotonic Tris-HCl buffer (0.3 M, pH 7.4). The haemolysate was prepared by suspending washed red blood cells with hypotonic buffer (Tris-HCl buffer 0.01 M, pH 7.4).

5.2.2.4 Biochemical estimation

The levels of lipid peroxidation, reduced glutathion, ascorbic acid, vitamin E and enzymes such as superoxide dismutase, glutathione peroxidase were estimated. protein was also estimated.

5.2.2.4.1 Estimation of protein

Protein was estimated by the method of Lowry et al., (1951), under section 3.1.4.3.5.
5.2.2.4.2  Superoxide dismutase (EC : 1.15.1.1, SOD)

Superoxide dismutase was assayed according to the method of Marklund and Marklund (1974) under section 4.2.4.1.

5.2.2.4.3  Glutathion peroxidase

Glutathione peroxidase was assayed by the method of Rotruck <i>et al.</i>, (1973) under section 4.2.4.3

5.2.2.4.4  Reduced glutathione

Reduced glutathione was determined by the method of Moron <i>et al.</i>, (1979) under section 4.2.5.1.

5.2.2.4.5  Vitamin C (Ascorbic acid)

Ascorbic acid was estimated by the method of Omaye <i>et al.</i>, (1979) under section 4.2.5.2

5.2.2.4.3  Vitamin E

Vitamin E was estimated by the method of Desai (1984) under section 4.2.5.3.

5.2.2.4.3  LPO

Lipid peroxidation was estimated by the method of Ohkawa <i>et al.</i>, (1979) under section 4.2.6.1
5.2.2.5 **Histopathology study**

Histopathological studies were done in hind limb joints of the animals. The tissue was fixed in 10% formalin, decalcified and embedded in paraffin blocks. Sections were stained with haematoxylin and eosin and examined under microscope and photomicrographs were taken (Gorden and Bradbury, 1990).

5.2.2.6 **Statistical analysis**

The statistical analysis was carried out using ONE WAY analysis of variance (ANOVA) followed by Dunnet’s T– test, P- values < 0.05 were considered as significant.

5.3. **Results**

5.3.1 **Anti-inflammatory activity**

5.3.1.1 **Carrageenan paw oedema**

Both *Ricinus communis* root extracts (RCA, RCM) *Cassia fistula* stem bark extracts (CFA and CFM) at doses (250 and 500mg/ kg /po )exhibited significant (P < 0.01) reduction in paw edema volume of rats. The percentage inhibition of extracts were shown in Table 9.

5.3.1.2 **Cotton pellet granuloma**

Both *Ricinus communis* root extracts (RCA, RCM) *Cassia fistula* stem bark extracts (CFA and CFM) at the dose level of 500 mg/kg/p.o., significantly (P < 0.001) reduced the weight of the cotton pellet granuloma in rats. The percentage inhibition of RCA was 33.68%, RCM was 43.78%, CFA
was 42.69%, CFM 22.31% and Diclofenac, the reference standard 50.42% as shown in Figure 3.

5.3.2 Antiarthritic evaluation

5.3.2.1 Complete freund’s adjuvant – Carrageenan model

5.3.3 Paw Volume

The paw volume was increased in Group II animals. A significant reduction (p<0.001) in paw volume was observed in RCA, RCM, CFA, CFM and Diclofenac treated rats compared to the induced Group II animals. The results were shown in Table 10.

5.3.4 Antioxidant estimation

Antioxidant levels in liver, plasma and haemolysate were shown in Table 11 and 12.

5.3.4.1 Lipid peroxidation

LPO level was significantly increased in the liver and plasma of the carrageenan challenged Group II animals when compared to control animals. The Lipid peroxide level of RCA, RCM, CFA, CFM and Diclofenac treated animals showed significant reduction (p<0.001) when compared to Group II animals.

5.3.4.2 Superoxide dismutase – SOD

SOD level of Group II animals was significantly decreased when compared to Group I animals. RCA, RCM, CFA, CFM and Diclofenac treated groups of animals showed significant (p<0.001) increase in the level of SOD when compared to Group II animals.
5.3.4.3 GPx Level

GPx level was significantly reduced in Group II animals when compared with Group I. RCA, RCM, CFA, CFM and Diclofenac treated animals showed significant (p<0.01) increase when compared to Group II animals.

5.3.4.4 Reduced glutathione

Reduced glutathione level was significantly reduced in Group II animals when compared with Group I animals. But the drug treated animals (RCA, RCM, CFA, CFM and Diclofenac) showed significant increase (p<0.001) when compared to Group II animals.

5.3.4.5 Vitamin C and Vitamin E

Vitamin C and Vitamin E levels were significantly reduced (p<0.001) in Group II animals when compared with Group I animals. The drug treated animals (RCA, RCM, CFA, CFM and Diclofenac) showed significantly increased when compared to Group II animals.

5.3.5 Histopathological studies

Plate 9 shows the histopathological changes of the hind limb joint of control, arthritic control and drug treated groups. The joints of the complete freund’s adjuvant – carrageenan (group II) treated rats were observed full of inflammation and fibrosis in synovial area. The joints of the RCA, RCM, CFA and CFM treated animal shows less inflammation in the synovial area and synovium present.
5.4 Discussion

In Indian System of Medicine (ISM), certain herbs are claimed to provide relief of pain and inflammation. The claimed therapeutic reputation has to be verified in a scientific manner.

The Ricinus communis root and Cassia fistula stem bark extracts possess significant anti-inflammatory effect in the acute and chronic anti-inflammatory model of inflammation in rats. Reactive oxygen species (ROS) generated endogenously or exogenously are associated with the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and aging process (Guyton et al., 1997, Halliwell and Gutteridge, 1999). Inflammation is a complex process and ROS play an important role in the pathogenesis of inflammatory diseases. (Conner and Grisham, 1996). Thus antioxidants which can scavenge ROS are expected to improve these disorders. Carageenan induced inflammation is a useful model to detect oral action of anti-inflammatory agents (Di Rosa et al., 1971). The development of oedema in the paw of the rat after the injection of carageenan is due to release of histamine, serotonin and prostaglandin like substances (Vinegar et al., 1969). The significant ameliorative activity of the extracts (RCA, RCM, CFA and CFM) and standard drug observed in the present study may be due to inhibition of the mediators of inflammation such as histamine, serotonin and prostaglandin. The carageenan assay is a good method for the comparative bioassay of anti-inflammatory agents. The present results indicate the efficacy of Ricinus communis root and Cassia fistula stem bark as an efficient therapeutic agent in acute anti-inflammatory conditions. The cotton pellet granuloma method
(D’Arcy et al., 1960) has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation. The fluid absorbed by the pellet greatly influences the wet weight of the granuloma. The results indicate that RCM and CFA has more anti-transudative and antiproliferative activity than RCA and CFM.

Free radicals have long been implicated as mediators of tissue injury in arthritic conditions (Bauerova et al., 1999). The granulocytes and macrophages accumulate in the affected area and produce large amount of superoxide and hydrogen peroxide radicals (Halley, 1993). The estimation of these reactive species in arthritic induced and drug treated animals help in assessing the antioxidant activity and indirectly the anti-arthritic potential of the plant drugs.

Measurement of paw oedema is the major factor in evaluating the degree of inflammation and therapeutic effect of the drugs. The complete freund’s adjuvant – carrageenan treated rats showed swelling around the ankle joints, it was considered to be the edema of the particular tissues. The swelling has been found to be increasing in the initial phase of inflammation and then becomes constant during the experiment. The extracts RCA, RCM, CFA, CFM and Diclofenac treated groups, reduced the swelling, may be due to the immunological protection.

LPO is a free radical released process that occurs in biological systems, eg. for the generation of lipid derived inflammatory mediators (Romero et al., 1998). The high levels of LPO induce diseases combined with inflammation, for instance rheumatoid arthritis. (Spiteller, 2003). The involvement of free radicals in various inflammatory conditions like synovitis
and RA were well documented (Merry et al., 1989, Halliwell et al., 1988). In the present study LPO level was increased in complete freund’s adjuvant – carrageenan treated rats. This indicates that the tissues are subjected to increased oxidative stress while reduction of LPO activity was observed in RCA, RCM, CFA, CFM and Diclofenac sodium treated animals.

Glutathione is an intra-cellular thiol rich tripeptide which plays a major role in cells and tissue structure (Meister, 1983). Low level of glutathione is indicated in arthritis. It is revealed in the present study that reduced glutathione content is lower in complete freund’s adjuvant – carrageenan induced animal. In RCA, RCM, CFA, CFM and Diclofenac treated animals showed elevated levels of glutathione content there by showing protective role of extracts in arthritis.

SOD, an enzymic antioxidant, catalytically scavenges the superoxide radical and thus provides the first line of defence against free radical damage. SOD activity is significantly reduced in complete freund’s adjuvant – carrageenan induced animals. Whereas the extracts RCA, RCM, CFA, CFM and Diclofenac sodium treated animals showed significant increase when compared with arthritic (complete freund’s adjuvant - carrageenan) animals.

Vitamin E acts as a major chain breaking antioxidant. Vitamin E level was decreased in complete freund’s adjuvant – carrageenan arthritic rats. Along with other antioxidants, Vitamin E has been shown to modulate the activity of the cyclooxygenase and lipoxygenase enzymes and also reduce the synthesis of pro-inflammatory prostaglandines (PG-2) and leukotrienes (LT B4
Vitamin E used as a supplement to arthritic patients has shown modest improvements.

Vitamin C, a cellular aqueous phase antioxidant has been shown to exert protection against oxidative stress (Chamundeeswari et al., 2003). Vitamin C level was decreased in complete Freund’s adjuvant treated animals. There is an increase in level of Vitamin C in the drug treated animals.

From the above results it can be concluded that the extracts RCA, RCM, CFA and CFM exhibited significant antiinflammatory and antiarthritic activities in experimental animal models.