Chapter 6

Bioactivities

6.1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that mainly targets the joints [1]. Both genetic and environmental factors are involved in the initiation and progression of the disease. Initiation of RA involves the activation of auto reactive T cells and the recruitment of these T cells along with other leukocytes into the joints. These leukocytes produce a variety of mediators of inflammation that induce synovial inflammation and eventually cause tissue damage in the joints. However, RA affects different people in different ways, in terms of the symptoms they have, how serious the symptoms are and how long the symptoms last. It has societal effect in terms of cost, disability and lost productivity. There are 150 varieties of arthritis affecting almost 150 million people in India. The second most affecting type of arthritis is Osteoarthritis (OA). A major distinction between OA and RA is that the former strikes the larger joints (knees, hips) first whereas latter first strikes the smaller joints [2]. OA is the breakdown of the joint articular cartilage which results in release of matrix components into the synovial fluid where they may become exposed to immune systems of the patients [3]. Close to 15 per cent of the general population could have one or other form of arthritis. RA is known to affect 1-2% of population and does not have any racial predilections. Every third person above the age of 70 years suffers from arthritis induced knee pain [4]. In people aged 15 to 45 years, women predominate by a ratio of 6:1, which is equal among patients in the first decade of life and in those more than 60 years old [5]. The causes behind this are complex and the exact pathology of disease is still to be understood. However, efforts are been made to understand the molecular mechanism for pathogenesis of RA [6].

Since ancient times our ancestors have used phytochemicals found in plants to curtail the inflammatory process. The emergence of today's pharmaceutical industry, in large part, has been based on natural products. It has been reported that proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukins (IL): IL-1β and IL-6 are important mediators of the disease perpetuation. Immune cells such as T cells also impart their role in the progress of the disease. Apart from the conventional treatment strategies using non steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) and glucocorticoids newer and safer drugs are continuously being searched. Alternative medicine is another therapeutic approach for treatment of the disease, which
include herbal and folklore medicines. Many plants and plant products are under scientific exploration to develop a novel therapeutic agent. In recent days, researchers are directed towards traditional system of medicine for the discovery of drugs that are long acting anti-inflammatory with minimum side effects. Although there is no ideal animal model for RA at this time, rat adjuvant arthritis shares many features of human RA [7], and the sensitivity of this model to anti-arthritic agents [8] support the view the adjuvant arthritis is the best available model of rheumatoid arthritis.

6.1.2. Safety profile evaluation of Herbals

The ethnobotanical uses of plants are diverse in traditional medical practices and the use of plants for medicinal purposes dates back to antiquity. Although the plant products are assumed to be safer than the synthetic medicines, a general assumption of this safety should not always be made as a plant may prove efficacious but would have low therapeutic index or safety margin. Drugs that survive the initial screening and profiling procedure must be carefully evaluated for potential risks before clinical testing is begun. While no chemicals can be certified as completely “safe” (free of risks), since every chemical is toxic at some level of dosage, it is usually possible to estimate the risk associated with exposure to the chemical under specified conditions, if appropriate tests are performed [9]. Toxicology is the science that deals with the adverse effect of chemicals on living organisms. Toxicology is concerned with the deleterious effects of chemical and physical agents on all living systems. In the biomedical area, however, the toxicologist is primarily concerned with adverse effects in humans resulting from exposure to drugs and other chemicals as well as the demonstration of safety or hazard associated with their use [10]. Determination of the dose response is crucially important since the dose response phenomenon is extremely important in toxicology, which is used to determine the minimum lethal dose of any experimental material [11].
6.2 EXPERIMENTAL

● MATERIALS AND METHODS

● Plant material

The fresh plants of *Abutilon indicum*, *Hygrophi*la auriculata* and *Trichosanthes dioica* were collected from the field area of Baryahi, Saharsa District Bihar, India, in January and May 2009. The plant specimens were authenticated by Prof (Dr.) Anjani Kumar Sinha, Principal, M L T Saharsa College, Saharsa, Bihar. A voucher specimen no SHC 55/01/2009, 56/05/09 and 57/05/09 has been deposited at the herbarium, Department of Botany, M L T Saharsa College, Saharsa- 852201.

● Preparation of extract

The plants *A. indicum*, *H. auriculata* and *T. dioica* were air-dried and powdered. 500 g of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with methanol for 72 hrs separately. Thereafter, methanolic extracts of each plants were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried. The yields of the methanolic extract were 13.2, 17.3 and 14.3 % w/w, respectively.

● Safe dose determination study

● Selection of animals

Swiss albino mice of either sex (25–30 g) were selected for this study. The animals were divided into the six groups of four animals each. The animals were kept in polypropylene cages and maintained at a temperature of 25 ± 2 C. The study has got the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the purposes of control and supervision of experiments on Animals).

● Dose levels

A single dose of extract of methanolic *A. indicum*, *H. auriculata* and *T. dioica* was administered in 0.3% carboxy-methyl cellulose (CMC) suspension at doses of 500, 1000, 2000, 4000 and 5000 mg/kg/b.wt, whereas the control group received the CMC suspension only for each plant extracts. The animals were allowed free access to water
and food. However, all the animals were deprived of food for 2 hr before and 4 hr after dosing.

- **Safety study of selected dose**

  - **Selection of animals**

    Wistar albino-rats of either sex weighing between 140-160 g used for this study. The animals were permitted free access to standard pelleted food and water. The animals are grouped in to four of 6 animals each. Control group (group 1) had 6 rats (3 males and 3 females) while group 2 was the low dose group with 6 rats (3 males and 3 females), group 3 was intermediate dose group with 6 rats (3 males and 3 females) and group 4 was the high dose group with 6 rats (3 males and 3 females).

  - **Dose levels**

    Three dose levels of methanolic extract of *A. indicum*, *H. auriculata* and *T. dioica* (100 mg/kg, 200 mg/kg and 400 mg/kg/b.wt) was given orally by gavage routinely (daily once) in appropriate quantity of water throughout the study period (28 days). The control group was allowed free access to water.

**6.2.2. ANTI-ARTHRITIC ACTIVITY (Complete Freund’s adjuvant induced arthritis)**

- **Chemicals**

  Freund’s adjuvant complete (CFA), Griess reagent system were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diclofenac sodium was procured from Ranbaxy lab’s New Delhi. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5-5 dithio-bis-2-nitrobenzoic acid (DTNB), Nitrobluetetrazolium (NBT), Ethylene diamine tetra-acetic acid (EDTA), xanthine, xanthine oxidase and Tris hydrochloride were purchased from SD Fine chemicals India. All other routine chemicals used in this investigation were of research grade.

- **Test samples and standards**

  Suspension of the methanolic extract of *A. indicum* (ABM), *H. auriculata* (HAM) and *T. dioica* (TDM) and diclofenac sodium was prepared in carboxy methyl cellulose (CMC,
0.3%) using distilled water. Gastric administration of all drugs was accomplished via oral route.

- **Animals**

Male Wistar rats (150–170 g) were used. They were kept in the Animal House of Faculty of Pharmacy, Integral University in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity 45–55 % with 12 h light/dark cycles after initial acclimatization for about 1 week. They had free access to standard rodent pellet diet and water *ad libitum*. The experimental study was conducted in accordance with the Institutional Animal Ethics Committee of the University.

- **Animal model (Complete Freund’s adjuvant induced arthritis)**

Adjuvant induced arthritic (AIA) is a model of chronic inflammation that exhibits several pathological changes similar to those occurring in rheumatoid arthritis. Adjuvant arthritis was induced by the subplanter injection of 0.1 ml Complete Freund’s adjuvant (1.0 mg of heat killed mycobacterium tuberculosis per ml of paraffin oil) [12, 13].

- **Evaluation of the severity of arthritis**

Paw volumes of injected (primary lesions) and non injected paws (secondary lesions) were measured on 7th, 14th, 21st and 28 days after injection. Animals were divided into five groups of six animals each. The body weight changes were observed on every week.

- Group I: Animals served as control.
- Group II: Arthritis-induced animals. Arthritis was induced by intradermal injection of 0.1 mL Freund's complete adjuvant to the rats hind paw.
- Groups III: were treated with standard drugs Diclofenac sodium 10 mg/kg, b.wt.
- Group IV: were treated with methanolic extract of HAM 100 mg/kg, b.wt
- Group V: were treated with methanolic extract of HAM 200 mg/kg, b.wt
- Group VI: were treated with methanolic extract of ABM 100 mg/kg, b.wt
- Group VII: were treated with methanolic extract of ABM 200 mg/kg, b.wt
- Group VIII: were treated with methanolic extract of TDM 100 mg/kg, b.wt
- Group IX: were treated with methanolic extract of TDM 200 mg/kg, b.wt
Biochemical assays

- Haematological analysis
The hematological parameters like hemoglobin (Hb), RBC, WBC, Platelets, ESR and rheumatoid factor (RF) were determined by usual standardized laboratory method [14].

- Preparation of cell-free extract of the knee joints
At the end of experiment animal were sacrifices by cervical dislocation. Arthritic and nonarthritic joints were removed and cut into small pieces and homogenized in 5 vol of 50 mM Tris HCl buffer, pH 7.4 containing 0.1 M NaCl and 0.1% Triton X-100 and 1 vol. of fine glass powder by using a mortar and pestle. The crude extract then was sonicated for 20 sec. The homogenate was centrifuged at 3,000 × g for 5 min, and the resulting supernatant was stored at -80°C until further analysis.

- Estimation of thiobarbituric acid reactive substances (TBARS)
The assay of TBARS was done according to earlier method [15], adapted to microtiter plates by bringing the final volume to 150 µl. In brief, tissue homogenate was prepared in 0.15 M KCl (5% w/v homogenate) and aliquots of 30 µl were incubated for 0 °C and 37 °C for 1hr. Subsequently, 60 µl of 28% w/v TCA was added and the volume was made up to 150 µl by adding 60 µl of distilled water followed by centrifugation at 3000xg for 10 min. The supernatant (125 µl) was taken and colour was developed by addition of 25 µl of 1% w/v TBA dissolved in 0.05 N NaOH and kept in boiling water bath for 15 min. The absorbance was read at 532 nm in a plate reader (Bio-Rad, U.S.A). The result was expressed in µmoles TBARS formed/hr/g tissue using a molar extinction coefficient of 1.56×10^5 M⁻¹ cm⁻¹.

- Reduced glutathione (GSH)
GSH was measured in the groups following the method described in [16]. Homogenized joint tissue (10% w/v in phosphate buffer pH 7.4) was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4 °C for 2 hrs. The contents were centrifuged at 2000xg for 15 min. 50 µl supernatant was added to 200 µl of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA (pH 8.9) followed by the addition 20 µl of
0.01M DTNB. The absorbance was read in a microplate reader at 412 nm and results are expressed as µg GSH/g tissue using a molar extinction coefficient of 13.6×10³ M⁻¹ cm⁻¹.

- **Total superoxide dismutase (SOD) activity**
  Total SOD were measured in joints as described in [17] adapted to microtiter plates by bringing the final volume to 100 µl. Reaction mixture consisted of 0.05M phosphate buffer (pH 7.4), 1mM Xanthine and 57µM NBT. After incubation at room temperature for 15 min., reaction was initiated by addition of 50 mU Xanthine. The SOD activity is expressed in Units/mg protein using a molar extinction coefficient of 4.02×10³ M⁻¹ cm⁻¹.

- **Catalase activity**
  Catalase activity in the joint tissues was assayed according to method described earlier [18] using H₂O₂ as substrate. The reaction was adjusted to multiwell flat bottom plates by reducing the final volume to 200 µl. Briefly reaction mixture consisted of phosphate buffer (0.01M, pH 7.0), distilled water and 10% homogenate (prepared in 0.1M phosphate buffer). Reaction was started by adding H₂O₂ (0.2M), incubated at 37°C for 1 min. and reaction was stopped by addition of dichromate: acetic acid reagent (1:3). The tubes were kept in a boiling water bath for 15min. and centrifuged for 10min at 1500 x g. The colour developed was read at 570 nm in a microplate reader. The enzyme activity was expressed as µmol H₂O₂ consumed/min/mg protein using a molar extinction coefficient of 43.6 M⁻¹ cm⁻¹.

- **Measurement of Nitric oxide (NO): Griess reaction**
  After the experiment, animals were sacrificed and the joint tissues were washed with PBS (pH 7.4) and placed on ice as method described earlier [19]. Briefly a 50µl sample was added with 100µl of Griess reagent and reaction mixture was Incubate for about 5-10 minutes at room temperature and protects it from light, the optical density was measured at 540 nm in microplate reader according to the reagent manufacturer’s protocol. Calculations were done after generating a standard curve from sodium nitrite in the same buffer as used for preparation of homogenate.
• **Measurement of cytokines level**

Levels of inflammatory cytokines TNF-α, IL-1β and IL-6, in the joints were determined by using commercially available cytokine ELISA kits (eBioscience and Cayman Chemical USA). Supernatants were removed and assayed in duplicate according to the manufacturer’s guidelines. Tissue cytokine concentrations were expressed as picograms of antigen per milligram of protein.

• **Histological examinations**

Rats were sacrificed at day 28th by cervical dislocation. Knee joints were removed and fixed in 4% formaldehyde. After decalcification in 5% formic acid, the samples were processed for paraffin embedding [20]. Tissue sections (5 µm thick) were stained with haematoxylin–eosin for light microscope examination.

**Protein content**

Protein was determined by Bradford method [21] using bovine serum albumin (BSA) as a standard.

• **Statistical Analysis**

Results are expressed as mean ±SEM. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's test for all parameters. The p-value <0.05 was considered statistically significant.

6.3. **RESULTS**

❖ **Safe dose determination study**

From safe dose determination studies it was observed that the administration of methanolic extract of *A. indicum, H. auriculata* and *T. dioica* to mice did not induce drug–related toxicity and mortality in the animals. The animals treated with methanolic extracts of *A. indicum, H. auriculata* and *T. dioica* were well tolerated and exhibited normal behavior up to 5 g/kg orally. All animals were alert with normal grooming, touch response, pain response and there was no sign of passivity, stereotypy, and vocalization. Their motor activity and secretory signs were also normal.
Safety profile studies of selected dose

Table 6.1: Effect of *A. indicum* methanolic extract on haematological parameters of rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th><em>A. indicum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/kg, b.wt.</td>
<td>100 mg/kg, b.wt.</td>
</tr>
<tr>
<td>RBC (×10^6/µL)</td>
<td>08.84 ± 0.53</td>
<td>09.13 ± 0.67</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>15.16 ± 1.02</td>
<td>15.54 ± 1.12</td>
</tr>
<tr>
<td>PLT (×10^3/µL)</td>
<td>796.4 ± 23.26</td>
<td>789.8 ± 30.48</td>
</tr>
<tr>
<td>WBC (×10^3/µL)</td>
<td>07.58 ± 1.14</td>
<td>07.62 ± 1.17</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *(n=6)*, Group 1: 0 mg/kg, b.wt, Group 2: 100 mg/kg, Group 3: 200 mg/kg, Group 4: 400 mg/kg body weight. No significant difference *(P<0.05)* were observed in haematological parameter between the treated and untreated groups of rats at the end of study (28 days).

Table 6.2: Effect of *H. auriculata* methanolic extract on haematological parameters of rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th><em>H. auriculata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/kg</td>
<td>100 mg/kg/b.wt</td>
</tr>
<tr>
<td>RBC (×10^6/µL)</td>
<td>08.43 ± 0.15</td>
<td>08.61 ± 1.07</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>15.17 ± 1.16</td>
<td>15.34 ± 1.01</td>
</tr>
<tr>
<td>PLT (×10^3/µL)</td>
<td>756.2 ± 29.34</td>
<td>791.7 ± 31.24</td>
</tr>
<tr>
<td>WBC (×10^3/µL)</td>
<td>07.83 ± 1.34</td>
<td>07.34 ± 1.41</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *(n=6)*, Group 1: 0 mg/kg, b.wt. Group 2: 100 mg/kg, b.wt. Group 3: 200 mg/kg, b.wt. Group 4: 400 mg/kg body weight. No significant difference *(P<0.05)* were observed in haematological parameter between the treated and untreated groups of rats at the end of study (28 days).
Table 6.3: Effect of *T. dioica* methanolic extract on hematological parameters of rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>T. dioica</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (×10^6/µL)</td>
<td>0.87 ± 0.43</td>
<td>0.86 ± 0.32</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>15.12 ± 1.02</td>
<td>15.39 ± 1.08</td>
</tr>
<tr>
<td>PLT (×10^3/µL)</td>
<td>796.4 ± 23.26</td>
<td>789.8 ± 30.48</td>
</tr>
<tr>
<td>WBC (×10^3/µL)</td>
<td>0.72 ± 1.04</td>
<td>0.63 ± 0.84</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. (n=6), Group 1: 0 mg/kg, b.wt, Group 2: 100 mg/kg, b.wt. Group 3: 200 mg/kg, b.wt. Group 4: 400 mg/kg body weight. No significant difference (P<0.05) were observed in haematological parameter between the treated and untreated groups of rats at the end of study (28 days).

- **Findings:** From the present results it was concluded that there is no significant alterations found in the haematological parameters by the treatment with the selected dose of each plant extracts when compared to untreated groups.

- **ANTI-ARTHRITIC ACTIVITY (Complete Freund’s adjuvant induced arthritis)**

- **Changes in body weight**

  The onset stage of AIA was associated with drastic body weight loss that continued till the end of experimental period, i.e., day 28 in the AIA rats (Table 6.4). Methanolic extract of *A. indicum* (ABM), *H. auriculata* (HAM) and *T. dioica* (TDM) and Diclofenac treatment proved beneficial for the AIA rats in regaining their original body weights and this trend continued throughout the experiment. Rats in the healthy control group showed a gradual increase in their body weight with respect to time. Rats in the ABM, HAM and TDM -treated group showed higher weight gain than did the Diclofenac-treated group.

- **Changes in paw volume**

  A significant increase in rat paw edema volume was observed in AIA rats when compared to the normal control rats. Treatment with diclofenac sodium at the dose of 10 mg/kg, body weight, and ABM, HAM, TDM at the doses of 100 mg/kg, 200mg/kg, body
weight respectively shows a significant réduction in rat paw edema volume when compared with the arthritic group. (Figure 6.1, 6.2 and 6.3).

- **Effects of A. indicum, H. auriculata and T. dioica treatment restored GSH and SOD levels**

  The Fig. 6.4, 6.5 and 6.6 A and B illustrate the changes in GSH level and SOD activity evaluated in the joints (day 28) in the experimental groups. The concentration of GSH was evaluated to estimate endogenous defences against hydrogen peroxide formation and SOD activity was measured to estimate endogenous defences against superoxide anions. A marked decrease in GSH (p<0.01) and SOD (p<0.001) concentrations were found in the joints of AIA rats. Treatment with ABM, HAM and TDM and diclofenac significantly inhibited reduction of GSH level and SOD level as compared to AIA group.

- **Effects of A. indicum, H. auriculata and T. dioica treatment decreased TBARS**

  The effect of ABM, HAM and TDM on TBARS level was measured to demonstrate the oxidative damage on lipids (Fig. 6.4-C, 6.5-C and 6.6-C). A significant increase (p<0.001) in TBARS level was observed in AIA group as compared to the control group. Treatment with ABM, HAM and TDM and diclofenac decreased TBARS level by inhibiting lipid peroxidation in the cartilage tissue.

- **Effects of A. indicum, H. auriculata and T. dioica on catalase activity**

  The activity of catalase decreased significantly in AIA group on the day 28 in the joints as compared to control group (Fig. 6.4-D, 6.5-D and 6.6-D). In this case too, treatment with ABM, HAM and TDM and diclofenac was significantly effective as compared to AIA group.

- **Effects A. indicum, H. auriculata and T. dioica on nitric oxide**

  Analysis of nitrite estimation is summarized in Fig. 6.4-E, 6.5-E and 6.6-E. A significant increase in nitrite was observed in AIA group as compared to control. The treatment with ABM, HAM and TDM and diclofenac declined the increase in the nitrite levels significantly as compared to the AIA group.
• **Haematological parameters**

After administration of CFA, there was significant decrease in RBC count, haemoglobin from normal levels and significant increase in total WBC count, and ESR levels above the normal. However, as shown in Table 6.5, 6.6 and 6.7. ABM, HAM and TDM and diclofenac are shown significant to increase the haemoglobin and RBC content when compare with the control group. However, HAM and diclofenac treated groups significantly decreased the total WBC count. The ESR count, which drastically increased in arthritic control group which was normalized by ABM, HAM and TDM and diclofenac treated groups.

• **A. indicum, H. auriculata and T. dioica suppresses IL-1β, TNF-α, IL-6 in RA rats**

Proinflammatory cytokines IL-1β, TNF-α, IL-6 have central role in the perpetuation of chronic inflammation and tissue damage during progression of RA. As shown in Fig. 6.7, 6.8 and 6.9, there was significant increase in the level of TNF-α (P<0.01), IL-1β (P<0.01) and IL-6 (P<0.01) in RA rats compared to the controls. Oral administration of ABM, HAM and TDM and diclofenac significantly down regulated the level of IL-1β, IL-6 and TNF-α as compared to AIA group on day 28.

• **Histopathology**

Histopathological examinations of knee joints were performed by the method of Belur et al., 1990 [22]. For histological examinations on the 28th day, the joints were isolated and preserved in 10% neutral buffered formalin. Histopathological observation of tissues was carried out in a Pathology laboratory, at Era Medical College, Lucknow (India). After fixation, the tissues were embedded in paraffin and clear in xylene and dehydrated in descending series of ethanol. At least four cross-sections were taken from each tissue of 5 μm thickness and stained with hematoxylin and eosin (H and E). Following two changes xylene washes of 2 min each, tissue sections were mounted with DPX mount. The slides were observed for histopathological changes and photomicrographs were taken using an Olympus BX-51 microscope system (Olympus, Japan). H&E staining was used to visualize and differentiate between tissue components in normal and in adjuvant induced arthritis (AIA). Histological evaluation of the joints in the AIA rats revealed marked
characteristic features of severe arthritis like synovial hyperplasia, massive mixed (neutrophil, macrophage, and lymphocyte) infiltration (Fig. 6.10 B) along with articular cartilage and bone erosion. No such arthritic features of inflammation and tissue destruction were evident in the joints of normal control group (Fig.6.10 A). Treatment with standard drug diclofenac sodium shows (Fig. 6.10 C) a normal joint architecture with no such arthritic features of inflammation and tissue destruction as seen in arthritic control rats. In the AIA rats treated with ABM (Fig. 6.10 D and E), HAM (Fig. 6.10 F and G) and TDM (Fig. 6.10 H and I), the degree of arthritis was significantly reduced with respect to reduction of cartilage damage, massive cell influx along with minimal evidence of inflammation. Besides, joint destruction and synovial membrane of the ABM, HAM and TDM-treated rats was like normal synovium as compared to the sections obtained from the joints of AIA rats. In the above group rats supplemented with ABM extract (in a dose of 200 mg/Kg body weight) histological examination of joints reveals nearly normal joint architecture showing significant improvement in the above arthritic condition.

6.4. DISCUSSION

From the results it is clear that the decrease in RBC count and haemoglobin level represents the anaemic condition in arthritic rats. Upon treatment with ABM, HAM and TDM for 28 days are remarkably counteracted RBC count and haemoglobin concentration and thus justifying their significant role in the arthritic conditions. The more important causes are the abnormal storage of iron in the reticulo endothelial system and synovial tissue and the failure of bone marrow respond to anaemia [23]. CFA administration leads to rise in total WBC count due to the stimulation of immune system against the invading antigens / due to the release of IL-1β inflammatory response, IL-1β increases the production of both granulocyte and macrophages colony stimulating factors [24, 25]. The significant decrease in WBC in ABM, HAM and TDM treated groups shows its immune modulation effect. The elevated level of ESR is observed in response of stress or inflammations in arthritic control group, which is remarkably counteracted by ABM, HAM and TDM restoring back to near normal thus justifying its significant role in arthritic conditions.
A link between inflammation and bone homeostasis has been attributed to the effects of cytokines such as IL-1β, TNF-α, IFN-γ, IL-6 and PGE2 that are abundantly expressed in patients with RA and in the arthritic joints of rat with collagen-induced arthritis. Blockade of these molecules resulted in a reduction of disease severity and bone resorption [26, 27], while IL-4 and IL-10 have potent anti-inflammatory effects and suppress cartilage and bone pathology in RA [28]. As treatment with ABM, HAM and TDM shifts the balance of cytokines toward a bone protecting pattern that acts to both lower levels of TNF-α, IL-1β and IL-6, hence, it is plausible to suggest that part of the beneficial anti-inflammatory and cartilage / bone protective effects of ABM, HAM and TDM may be mediated through the inhibition of proinflammatory cytokines. This, in turn, would lead to reduced production of free radicals and subsequent damage.

During RA, lipid peroxidation is considered a critical mechanism of the injury [28]. The large amount of TBARS found is consistent with the occurrence of damage mediated by free radicals [29]. Cell damage can be prevented by detoxification of these free radicals, which eventually would prevent the progress of lipid peroxidation. In the present study, we found that AIA caused a significant increase in lipid peroxides and depletion in GSH and SOD [30, 31]. A reduction in GSH may impair H$_2$O$_2$ clearance and endorse OH$^-$ formation, thus increasing the free radical load that results in the disruption of homeostasis [32]. ABM, HAM and TDM treatment increased the activities of antioxidant enzymes significantly in the AIA group. Our results clearly indicate that the protective role of ABM, HAM and TDM was mediated via its antioxidant effect through the suppression of lipid peroxidation and boosting the antioxidant defence system.

Nitric oxide (NO) is an important signalling molecule, produced as part of the inflammatory response from activated cells and macrophages [33]. Inflammatory cytokines in chondrocytes induced excessive production of NO that has been implicated in the induction of apoptosis in chondrocytes [34]. Therefore, agents that hamper excessive NO production may have beneficial therapeutic effects in arthritis by blocking degradation of cartilage [35]. In the present study, increased NO level has been detected in arthritic group similar with those previously reported in synovial fluids of patients with rheumatoid arthritis [36]. Treatment with ABM, HAM and TDM produced a significant reduction in nitric oxide level.
This decrement and the other biochemical parameters were reflected in histological analysis, confirming the protective effects of the ABM, HAM and TDM. The higher number of infiltrating cells, extensive bone degradation and synovial hyperplasia which are hallmarks of RA was found in AIA while treatment with ABM, HAM and TDM was able to reverse the histological findings to normal.
Table 6.4: Influence of *A. indicum, H. auriculata* and *T. dioica* on body weight in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g) Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Control</td>
<td>156.00 ± 5.78</td>
</tr>
<tr>
<td>AIA</td>
<td>158.30 ± 5.94</td>
</tr>
<tr>
<td>AIA+ABM (100mg)</td>
<td>149.20 ±5.31</td>
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<td>AIA+ABM (200mg)</td>
<td>155.10 ±5.86</td>
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<td>152.40 ±5.76</td>
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<td>AIA+HAM (200mg)</td>
<td>152.80 ±4.80</td>
</tr>
<tr>
<td>AIA+TDM (100mg)</td>
<td>151.40 ±5.12</td>
</tr>
<tr>
<td>AIA+TDM (200mg)</td>
<td>154.10 ±5.70</td>
</tr>
<tr>
<td>AIA+Diclo (10mg)</td>
<td>150.60 ±4.54</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM of (n=6) rats.
Table 6.5: Effect of *A. indicum* methanolic extract in haematological parameters in control and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>AIA</th>
<th>AIA +ABM (100mg)</th>
<th>AIA +ABM (200mg)</th>
<th>AIA + Diclo (10mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>8.98±0.26</td>
<td>7.56±0.18**</td>
<td>8.78±0.29#</td>
<td>8.72±0.21##</td>
<td>9.12±0.14##</td>
</tr>
<tr>
<td></td>
<td>(millions/mm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>7.95±0.65</td>
<td>12.96±0.28**</td>
<td>9.26±0.39#</td>
<td>7.89±0.18##</td>
<td>8.58±0.35#</td>
</tr>
<tr>
<td></td>
<td>(thousands/mm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR(mm/hr)</td>
<td>3.76±0.23</td>
<td>10.98±0.29***</td>
<td>5.78±0.32##</td>
<td>3.96±0.26##</td>
<td>5.46±0.10##</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>14.76±1.38</td>
<td>9.65±1.96**</td>
<td>11.50±1.18##</td>
<td>13.92±1.92##</td>
<td>13.78±1.30##</td>
</tr>
<tr>
<td>Platlets</td>
<td>2.46±0.32</td>
<td>3.52±0.85**</td>
<td>2.82±0.79##</td>
<td>2.34±0.14##</td>
<td>2.58±0.28##</td>
</tr>
<tr>
<td></td>
<td>(lakhs/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF (IU/ml)</td>
<td>---</td>
<td>48.95±0.82**</td>
<td>34.33±1.24##</td>
<td>31.39±1.08##</td>
<td>31.27±1.87##</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM of (n=6) rats. ** (p<0.01), *** (p<0.001) vs. Control, # (p<0.05), ## (p<0.01) vs. AIA.
Table 6.6: Effect of *H. auriculata* methanolic extract in haematological parameters in control and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>AIA</th>
<th>AIA+HAM (100mg)</th>
<th>AIA +HAM (200mg)</th>
<th>AIA + Diclo (10mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (millions/mm³)</td>
<td>8.98 ± 0.26</td>
<td>7.56 ± 0.18**</td>
<td>8.34 ± 0.12#</td>
<td>8.67 ± 0.18##</td>
<td>9.12 ± 0.14##</td>
</tr>
<tr>
<td>WBC (thousands/mm³)</td>
<td>7.95 ± 0.65</td>
<td>12.96 ± 0.28**</td>
<td>8.96 ± 0.16#</td>
<td>7.96 ±0.34##</td>
<td>8.58 ± 0.35#</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>3.76 ± 0.23</td>
<td>10.98±0.29***</td>
<td>6.70 ± 0.18##</td>
<td>4.18 ±0.31##</td>
<td>5.46 ± 0.10##</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>14.76±1.38</td>
<td>9.65 ± 1.96**</td>
<td>12.25±1.55##</td>
<td>14.12±0.73##</td>
<td>13.78±1.30##</td>
</tr>
<tr>
<td>Platlets (lakhs/ml)</td>
<td>2.46 ± 0.32</td>
<td>3.52 ± 0.85**</td>
<td>2.76 ±0.37##</td>
<td>2.52 ±0.22##</td>
<td>2.58±0.28##</td>
</tr>
<tr>
<td>RF (IU/ml)</td>
<td>----</td>
<td>48.95 ±0.82**</td>
<td>33.27±1.58##</td>
<td>31.55±1.10##</td>
<td>31.27±1.87##</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of (n=6) rats. ** (p<0.01), *** (p<0.001) vs. Control, # (p<0.05), ## (p<0.01) vs. AIA.
Table 6.7: Effect of *T. dioica* methanolic extract in hematological parameters in control and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>AIA</th>
<th>AIA+TDM (100mg)</th>
<th>AIA+TDM (200mg)</th>
<th>AIA+Diclo (10mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (millions/mm³)</td>
<td>8.98 ± 0.26</td>
<td>7.56 ± 0.18**</td>
<td>8.92 ± 0.25#</td>
<td>8.84 ± 0.33##</td>
<td>9.12 ± 0.14##</td>
</tr>
<tr>
<td>WBC (thousands/mm³)</td>
<td>7.95 ± 0.65</td>
<td>12.96 ± 0.28**</td>
<td>10.38 ± 0.33#</td>
<td>8.16 ± 0.27##</td>
<td>8.58 ± 0.35#</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>3.76 ± 0.23</td>
<td>10.98 ± 0.29***</td>
<td>6.18 ± 0.11##</td>
<td>4.02 ± 0.16##</td>
<td>5.46 ± 0.10##</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>14.76 ± 1.38</td>
<td>9.65 ± 1.96**</td>
<td>12.17 ± 1.84##</td>
<td>14.36 ± 1.30##</td>
<td>13.78 ± 1.30##</td>
</tr>
<tr>
<td>Platlets (lakhs/ml)</td>
<td>2.46 ± 0.32</td>
<td>3.52 ± 0.85**</td>
<td>2.64 ± 0.29##</td>
<td>2.30 ± 0.35##</td>
<td>2.58 ± 0.28##</td>
</tr>
<tr>
<td>RF (IU/ml)</td>
<td>----</td>
<td>48.95 ± 0.82**</td>
<td>35.30 ± 1.82##</td>
<td>31.76 ± 2.06##</td>
<td>31.27 ± 1.87##</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM of (n=6) rats. ** (p<0.01), *** (p<0.001) vs. Control, # (p<0.05), ## (p<0.01) vs. AIA.
Figure 6.1: Effect of *A. indicum* in paw volume of control and experimental animals.

Figure 6.2: Effect of *H. auriculata* in paw volume of control and experimental animals.

Figure 6.3: Effect of *T. dioica* in paw volume of control and experimental animals.
Figure 6.4: Effect of methanolic extract of *A. indicum* (ABM) treatment on (A) GSH level, (B) SOD activity, (C) lipid peroxidase, (D) catalase activity and (E) articular nitrite in joints of rats. Data are expressed as Mean ± SEM of (n=6) rats. ** (p<0.01), *** (p<0.001) vs. control, # (p<0.05), ## (p<0.01), ### (p<0.01) vs. AIA.
Figure 6.5: Effect of methanolic extract of H. auriculata (HAM) treatment on GSH level, SOD activity, lipid peroxidase, catalase activity and articular nitrite in joints of rats. Data are expressed as Mean ± SEM of 6 rats. ** (p<0.01), *** (p<0.001) vs. control, # (p<0.05), ## (p<0.01), ### (p<0.01) vs.AIA.
Figure 6.6: Effect of methanolic extract of *T. dioica* (TDM) treatment on GSH level, SOD activity, lipid peroxidase, catalase activity and articular nitrite in joints of rats. Data are expressed as Mean ± SEM of 6 rats. ** (p<0.01), *** (p<0.001) vs. Control, # (p<0.05), ## (p<0.01), ### (p<0.01) vs. AIA.
Figure 6.7: Effects of *A. indicum* methanolic extract on cytokine levels were measured in rat joint exudates and concentration was expressed in pg/ml of (a) tumor necrosis factor-α (TNF-α) (b) interleukin-1β (IL-1β), (c) interleukin-6 (IL-6). Data are expressed as the mean ± SEM of *n* = 6 animals per group. ** *p* < 0.01 versus control group, # *p* < 0.05, ## *p* < 0.01, ### *p* < 0.001 versus AIA group.

Figure 6.8: Effects of *H. auriculata* methanolic extract on cytokine levels were measured in rat joint exudates and concentration was expressed in pg/ml of (a) tumor necrosis factor-α (TNF-α) (b) interleukin-1β (IL-1β), (c) interleukin-6 (IL-6). Data are expressed as the mean ± SEM of *n* = 6 animals per group. ** *p* < 0.01 versus control group, *p* < 0.05, ## *p* < 0.01, ### *p* < 0.001 versus AIA group.
Figure 6.9: Effects of *T. dioica* methanolic extract on cytokine levels were measured in rat joint exudates and concentration was expressed in pg/ml of (a) tumor necrosis factor-α (TNF-α) (b) interleukin-1β (IL-1β), (c) interleukin-6 (IL-6). Data are expressed as the mean ± SEM of *n* = 6 animals per group. **p<0.01 versus control group, *p<0.05, ##p<0.01, ###p<0.001 versus AIA group.

![Figure 6.9](image)

**Figure-6.10** Histological images of joint tissues at the magnification of 100X:
A- Normal control (Normal saline 1.0 ml/Kg), B- Arthritis control (Freund’s adjuvant CFA- 0.1 ml), C- Diclofenac sodium (10 mg/Kg) treated, D- ABM extract (100 mg/Kg) treated D-I, E- ABM extract (200 mg/Kg) treated D-II, F- HAM extract (100 mg/Kg) treated D-I, G- HAM extract (200 mg/Kg) treated D-II, H- TDM extract (100 mg/Kg) treated D-I, I- TDM extract (200 mg/Kg) treated D-II. The Arrow denotes erosive changes in cartilages from treated and non-treated rats i.e. Freund’s adjuvant-induced Arthritis-control rats.
6.5. References


Comparison of the Up-and Down conventional LD₅₀ and fixed dose Acute Toxicity procedure. Fundamental and Chemical Toxicology, 33, 223-231.


