CHAPTER 6: DISCUSSION

Freund’s Complete Adjuvant (FCA) was used to induce arthritis in rats to investigate the anti-arthritic and acute anti-inflammatory effect of petroleum ether, chloroform, methanol, ethanol and aqueous extracts with its potent extracts formulations like SHF-A, SHF-B, SHF-C and SHF-D of C. zedoaria root in arthritic rats. This study was designed as per the US- FDA guideline for industrial preclinical evaluation of anti-arthritis.

In the present study, clinical and behavioral aspects were considered for the evaluation of anti-arthritic activity which has been proposed as a common animal model for rheumatoid arthritis. Evaluation of acute anti-inflammatory activity of C. zedoaria root extracts were carried out by sub-planter injection of carrageenan and histamine induced rat paw edema.

In the present study, the efforts were made to elucidate possible mechanism of action that has been claimed by folk and traditional use of C. zedoaria root in crippling arthritis and frozen joints. The first phase was associated with acute inflammation and systemic effect on liver from 1st day to 4th day. The second phase begins on 7th day and lasts to 12th day with acute inflammation and periarthritis remission. The third phase was being observed from 12th to 28 days with chronic inflammation, periarthritis and osteogenic activity. The last phase of the arthritis includes permanent articular deformity and inflammation from 35th day onwards.

Hence, the present study of anti-arthritic study was carried out up to 42 days of post-inoculation through FCA injections in the tibiotarsal joint of rats. FCA injected joints produced unilateral arthritis in 100% of rats showing significant swelling within 24 hours in control rats.10,125,165

In the present study, C. zedoaria root powder was extracted by successive extraction method with petroleum ether, chloroform, methanol and ethanol. The aqueous extract was
obtained by the cold maceration process using the dried ethanol marc. All extracts were subjected to preliminary phytochemical study which revealed that, the different extract of *C. zedoaria* root showed the presence of different active constituents such as carbohydrates, proteins, amino acids, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolic compound. Table: 3.

Acute oral toxicity studies of each extract of *C. zedoaria* root showed that no toxic effects were observed at the dose of 2000 mg/kg body weight. Hence, $1/10^{th}$ (200 mg/kg) and 1/5 (400 mg/kg) of the lethal dose were selected as effective dose for further anti-arthritic and acute inflammatory activities.

**Anti-arthritis Activity of Root Extracts of *C. zedoaria***

Chronic inflammation in rat joint is manifested as a progressive increase in the volume of the FCA injected paw. It is noteworthy that the inhibitory effects of *C. zedoaria* root extracts on rat paw edema were observed in drug and extract treated groups. Reduction in paw edema was observed in standard-I, standard-II, petroleum ether, chloroform and ethanol extract of *C. zedoaria* root from third day to last day of study. However, methanol and aqueous extract treated groups at both dose showed no significant reduction in rat paw edema.

The body weight of rats used as an indirect index in restoration of health suggests that the decrease in the body weight during inflammation or disease condition is due to deficient absorption of nutrients through the intestine. The treatment with anti-inflammatory drugs normalizes the process of absorption$^{190-191}$. In the present study, the control group showed decline in the body weight from 14 days to last day of study. The standard-I, standard-II,
petroleum ether, chloroform and ethanol extract groups restored the body weight but showed decline in methanol and aqueous extract treated groups.

The haematological profile in arthritic condition shows reduction in RBC, haemoglobin and lymphocytes whereas increase in the WBC count and ESR level\textsuperscript{10}. It is proposed that the reduction in Hb count during arthritis was due to the reduced erythropoietin levels, due to decreased response of the bone marrow and premature destruction of red blood cells. Similarly, an increase in the ESR is attributed to the accelerated formation of endogenous proteins such as fibrinogen and a/b globulin, and rise in the ESR level which indicates an active but obscure disease. Moderate increase in the WBC count due to IL-1\textbeta mediated rise in the respective colony-stimulating factors.

In the present study, significant haematological alterations such as Hb, ESR, RBC, WBC, were observed in the control group, compared with normal group. But recovery was observed in the standards and extract treated groups. Methanol and aqueous extracts treated groups could not recover the haematological alterations induced by FCA in rats.

The toxicity in liver, kidney and heart are the most common adverse effect with nonsteroidal anti-inflammatory drugs, diseases modified anti-rheumatic drugs, steroids and biological agents\textsuperscript{13}.

The biochemical estimations of aspartate amino transferase (AST), alkaline amino transferase (ALT), blood urea nitrogen (BUN), uric acid, creatinine (CRE) and total protein were carried out to detect the toxic effect on the liver and kidney. In the present study, no significant changes were observed in biochemical parameters after 42 days of drug treatment compared with normal group.

Serum nitric oxide synthesis is produced by inducible nitric oxide synthase (iNOS) that has been demonstrated in rheumatoid arthritis\textsuperscript{150-151}. It has been expressed by several types of cells including macrophage, neutrophils, endothelial cells, chondrocytes and
synovial fibroblast. Increased level of nitric oxide metabolic products such as nitrate and nitrite were detected in serum, urine and synovial fluid and their concentration was related to disease progression\textsuperscript{152}.

In the present study, serum nitric oxide significantly increased in the control group but on the other hand serum nitric oxide level was reduced in all drug treated groups except aqueous 400 mg/kg when compared with the control group. These findings confirm that the presence of nitric oxide in rheumatoid arthritis and the inhibitory effect of treatment on nitric oxide synthesis would explain the possible mechanism of anti-arthritic activity.

In arthritic condition, a number of inflammatory mediators released from the site of injury caused vasodilatation. It has been hypothesized that Evan blue forms a complex bound with the large plasma proteins. The Evan’s blue has the capacity to pass through the enlarged endothelial gaps from where it can escape into interstitial spaces. The amount of Evan’s blue dye present in synovial capsule can provide the relative index of vascular permeability. In the present study, a significant augmentation in extravasations of Evans blue was observed in control group. The infiltration inhibitory effect of petroleum ether, chloroform, methanol and ethanol root extract of \textit{C. zedoaria} was observed at both doses. However, aqueous extract at both doses showed non significant inhibitory effect. The above data indicates that petroleum ether extract showed more significant anti-arthritic effect by decreasing endothelial gaps and vascular permeability better than Indomethacin treated group.

It has also been suggested that FCA-induced rheumatoid arthritis has a wide spread effect on physiological homeostasis due to the severe discomfort in animals. Behavioral approach to the arthritic rats that has been proposed as an animal model for chronic pain were made up to day 42 for the conformed status of disease. Behavioral observations like latency time to explore, ambulatory, rearing, grooming\textsuperscript{157-158}, urination, and defecation
were made over the 42 days post-inoculation period; The latency time to explore in FCA-induced arthritic rats has shown gradual delay in exploration ability. Treatment with Indomethacin and Rumalaya forte has shown an appreciable and significant decrease in latency time to explore during 14 to 42 days. Extract-treated groups improved the condition by decreasing latency time to explore during 14 days throughout the study. However, latency time to explore was identical in methanol and aqueous extract treated groups compared with control group.

Control group showed gradual decrease in ambulatory and rearing behavior during 3 to 42 days of the study. However, all drug and extract treated groups showed decrease in ambulatory and rearing behavior on the 3rd day and improvement in mobility and spontaneous condition during the 7th day upto 28 days, but complete reversal of ambulatory behavior was observed during 35 to 42 days.

The grooming effects by arthritis were observed during 3 to 14 days in normal group but the control group showed an increase in grooming effect that was observed during 3 to 28 days and recovery was observed on day 42. In all drug-treated groups, grooming effect by arthritis was observed during 3 to 21 days, and showed improvement during 28 to 42 days.

The angiogenic and anxiolytic effects of drug treatment on FCA-induced arthritic rats were studied by considering the frequency of urination and defecation in five minutes of exploratory. This fear was due to arthritis induced anxiety in the animals when placed in an open field. The ultimate manifestation of anxiety in the animals is exhibited by decrease in the motor activity. Anxiolytic agents are expected to increase the motor activity, which were measured by frequency of urine and defecation during observation^{124}.

Urine frequencies were reduced in 3 days but elevation in the frequency of urinations was observed on 7 to 28 days. In the control group, higher frequency of urine was recorded
during the observation period. However, drug-treated animal showed reduced urine frequency during 14 to 42 days except methanol 400 mg/kg and aqueous groups.

Defecation frequency was higher in the control group during the observation period, but there was significant decrease in defecation frequency in standard-I and petroleum ether during 14 to 42 days. However, standard-II, chloroform, methanol and aqueous treated group showed decreased frequency of defecation during 21 to 42 days of study.

Radiography is widely accepted as the gold standard in assessing structural joint damage associated with rheumatoid arthritis and is therefore considered as an essential parameter in evaluating the efficacy of experimental therapeutics. In present study, the radiographic examination revealed the presence of severe soft tissue swelling, but not the subsequent destruction of bones, cartilages, and narrowing of the joint spaces in the ankle joint of control, methanol and aqueous groups on day 42. However, standard-I, standard-II, petroleum ether chloroform and ethanol extract showed significant reduction in soft tissue swelling, among these extracts petroleum ether 200 mg/kg showed high reduction in soft tissue swelling of arthritic joint near to Indomethacin-treated group.

Histopathological examination of animals treated with standard-I, standard-II, petroleum ether, chloroform and ethanol extract of *C. zedoaria* root have shown protection against abnormality and deformation of digits and toes, indicating their applicability in the last phase of the arthritis and also improving its protective efficacy against permanent deformation161,162.

On the basis of histopathological examination control group showed marked joint damaged compared with normal joint whereas petroleum ether, chloroform and ethanol treated joints showed mild damaged. However, methanol and aqueous extract treated joints shown marked damaged near to the arthritic control group.
The difference in organ to body weight ratio between drug treated groups are often accompanied by differences in body weight between these groups. Organ-to-body weight ratios are predictive for evaluating the organ toxicity study. This evaluation has shown that analysis of organ-to-body weight ratios is predictive to evaluate spleen, thymus and adrenal gland. In the present study, significant changes were observed with the reduced weight of thymus and the gain weight of spleen and adrenal glands in the control group compared with normal group. In standard-I, standard-II, petroleum ether, chloroform and ethanol extract treated groups showed no significant changes in spleen, thymus gland, and adrenal gland. The weight of spleen increased and the weight of thymus reduced significantly. Significant change in the adrenal gland was observed in aqueous group.

**Acute Anti-inflammatory Activity:**

The present study, investigates the inflammation inhibitory effect of petroleum ether, chloroform, methanol, ethanol and aqueous extracts of *C. zedoaria* root. The most commonly used animal model for acute inflammation is carrageenan and histamine-induced rat paw edema. Carrageenan induced inflammation is a biphasic phenomenon\(^{192}\). The first phase of edema is attributed to release of histamine and 5-hydroxytryptamine. Plateau phase is maintained by kinin like substances and second accelerating phase of swelling is attributed to prostaglandin like substances. The knowledge of these mediators involved in different phases is important for interpreting mode of drug action.

In the present study, the petroleum ether, chloroform and ethanol extracts of *C. zedoaria* showed significant reduction in paw edema from 2 to 6 hours at both doses. It is suggested that curcumin produces an anti-edematous effect during the second phase, similar to Indomethacin. Therefore, our results confirm that the mechanism of the anti-inflammatory effect of curcumin involves reduction of prostaglandins through inhibition of cyclooxygenase. The antiedematous effect of methanol and aqueous extract of *C. zedoaria*
showed a delayed onset (6 h). In addition, the efficacy of petroleum ether, chloroform and ethanol extract of *C. zedoaria* was comparable to that of Indomethacin and Rumalaya forte with a longer duration of action that showed significant reduction in paw edema volume in carrageenan and histamine induced inflammation.

**Separation of Active Constituents and Characterization of Petroleum Ether Extract of *C. zedoaria* Root**

Separation, purification and characterization are the new tool for herbal drug to identify the active moiety in the plant extract. The present study was to investigate the separation, purification and characterization of active constituents which are responsible for antiarthritic and acute anti-inflammatory activity. Petroleum ether extract of *C. zedoaria* root showed highly potent activity.

Qualitative analysis of the extract was performed by chemical test and further was supported by thin layer chromatography, column chromatography, high performance thin layer chromatography and high performance liquid chromatography. Separated crude curcuminoid obtained from petroleum ether extract of *C. zedoaria* by using preparative thin layer chromatography was compared with standard curcuminoid by using solvent system chloroform : benzene : methanol at the ratio of 45:45:10. The curcuminoid was further fractionated by column chromatography. 150 fractions were obtained in the solvent system combined with chloroform and methanol at different ratios. In fraction 13-22 single spot was observed which was identified by TLC, HPLC and HPTLC that was considered as separated compound curcumin when compared with standard curcumin. The Rf value of separated compound was near to standard curcumin. Separated fraction was analyzed by preparative TLC and compared with standard. All separated compounds were recrystallized and observed for its physical property and chemical property then subjected to spectroscopy: UV, HPLC, HPTLC, FTIR, LCMS and \(^1\)HNMR for analysis. On the basis of
spectroscopy data the separated compound was curcumin which plays a major role in the
treatment of arthritis and inflammation

**Development of Single Herbal Formulation from Potent Extracts of *C. zedoaria* Root
and its Pharmacological Effect in Arthritic and Acute Inflammation**

Pharmacological evaluation of root extract of *C. zedoaria* revealed that petroleum ether, chloroform and ethanol extract showed potent extract for anti-arthritic and acute anti-inflammatory activity. On this basis, these extracts were formulated as SHF-A, SHF-B, SHF-C and SHF-D by using standard procedure.

Acute toxicity study of the formulations showed no toxic effects at higher dose of 2000 mg/kg b.w. Hence, 1/10th of this lethal dose were selected for further anti-inflammatory and acute inflammatory activity.

FCA-induced rat paw edema showed progressive increase in paw edema in control groups as compared to the normal group. However, all formulations reduced the paw edema from the 3rd day to 42nd day. However, SHF-B showed less reduction in rat paw edema from 3rd to 7th day compared to another formulation treated groups.

Body weight and haematological change such as RBC, haemoglobin, WBC count and ESR level were recovered with all formulations except SHF-B compared with the control group.

Changes in biochemistry parameters such as ALT, AST, BUN, Cr, uric acid, and total proteins were not found in any groups. All the values were near to normal group. There were no toxic effect on the liver, kidney and heart.

Inhibitory nitric oxide level and vascular permeability were observed in formulations treated groups compared to control group whereas SHF-B treated group showed less inhibitory effect on nitric oxide level and vascular permeability.

**Behavioral Approach**
Latency time to explore were increased and decreased ambulatory and rearing was observed in all groups at 3 day but, in standard-I, standard-II, SHF-A, SHF-B, SHF-C and SHF-D treated groups recovery were observed compared with 0 day. Rearing was not recovered in SHF-B treated groups and compared with 0 day. Increased grooming was found during 3 to 14 days in normal groups, all formulations -treated groups increased grooming during 3 to 21 days, but decreased during 28 to 42 days. However, in SHF-B grooming increased on 3 day to last day of study compared with 0 day. All formulations decreases the anxiety behavior includes urination and defecation but, SHF-B showed less effective in anxiety behavior.

Radiography examination of rat joints indicates that formulations-treated groups, showed significant reduction in soft tissue swellings except SHF-B groups showed a slight reduction in rat joint swelling compared with control joint.

Histopathology of rat joints showed that formulation SHF-A, SHF-C, and SHF-D treated groups showed marked protection against the hind paw tissue injury. However, SHF-B treated group showed a slight protective effect on FCA-induced arthritis in rat joints compared with control.

The present study investigates the organ weight. Organ-to-body weight ratio was carried out on the last days of the experiment. In formulations treated groups, no significant changes were observed in the spleen, thymus and adrenal glands that were near to normal group.

Carrageenan induced rat paw edema were selected for acute inflammatory activity. The SHF-A, SHF-C and SHF-D treated group showed reduction in paw edema, compared with the control group. Hence all formulations treated group SHF-A, SHF-C and SHF-D showed inhibitory effect in carrageenan induced rat paw edema.

Histamine induced rat paw edema showed significant (p< 0.001) reduction in the paw edema at 200 mg/kg dose of SHF-A, SHF-B, SHF-C and SHF-D.
Phytochemical data revealed that petroleum ether, chloroform and ethanol extracts of *C. zedoaria* showed presence of steroids, terpenoids, glycosides, alkaloids, tannins and phenolic compounds (curcuminoid). It was hypothesized that anti-arthritic and acute anti-inflammatory activity of petroleum ether, chloroform and ethanol extracts may be due to the presence of these active constituents. It has been well reported in literature that curcuminoid (curcumin, demethoxycurcumin and bisdemethoxycurcumin) inhibit TNF-induced NF-kB activation. Curcumin modulates the inflammatory response by down regulating the activity of cyclooxygenase-2 (COX-2), lipoxigenase, and inducible nitric oxide synthase (iNOS) enzymes and inhibits the production of the inflammatory cytokines. Curcumin suppresses the NF-kB activation and proinflammatory gene expression by blocking phosphorylation of inhibitory factor I kappa B kinase (IκB). Suppression of NF-kB activation subsequently by curcumin down regulate the COX-2 and iNOS expression, inhibiting the inflammatory process\textsuperscript{21-23}.

Steroids block allergic reactions and reduce the symptoms of itching, swelling and redness of skin and synthesis of certain enzymes that reduced inflammation and also suppress the immune system. One of the most effective families of natural product for their medicinal value is terpenoids have been used as anti-inflammatory which reduced production of Prostaglandin E\textsubscript{2} (PG\textsubscript{2}) and also suppress the NF-kb and iNOS\textsuperscript{22}.

Petroleum ether, chloroform and ethanol extracts of *C. zedoaria* roots and its formulation has beneficial effects in FCA induced arthritis in rats and carrageenan and histamine induced acute inflammation in rat paw edema may be due to the presence of these active constituents’ curcuminoid, terpenoids and steroids.

Accelerated stability studies of formulations were stable in both the environments that are at room temp and 40 °C temp up to three months.