EVALUATION OF ANTI-ARTHRITIC ACTIVITY OF THE ETHANOLIC EXTRACT OF *MIMOSA PUDICA LINN.* IN COMPLETE FREUND'S ADJUVANT INDUCED ARTHRITIS IN WISTAR RATS

Dissertation submitted to

THE TAMIL NADU DR.M.G.R.MEDICAL UNIVERSITY, CHENNAI

In partial fulfillment for the award of the degree of

MASTER OF PHARMACY

in

PHARMACOLOGY

by

Keerthi Kanimozhi K

Register No: 261525003

Under the Guidance of

Dr. P.Amudha, M. Pharm., Ph.D

Assistant professor

DEPARTMENT OF PHARMACOLOGY

C.L.BAID METHA COLLEGE OF PHARMACY

(AN ISO 9001-2008 CERTIFIED INSTITUTION)

CHENNAI – 600097

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Phone : 24960151, 24962592, 24960425
e-mail : clbaidmethacollege@gmail.com
Website : www.clbaidmethacollege.com

C.L. Baid Metha College of Pharmacy

An ISO 9001:2008 approved institution

Jyothi Nagar, Old Mahabalipuram Road,
Thorapakkam, Chennai - 600 097.

Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai.
Approved by Pharmacy Council of India, New Delhi, and
All India Council for Technical Education, New Delhi

Dr.P.AMUDHA, M.Pharm., Ph.D.,
Assistant Professor
Department of Pharmacology,

CERTIFICATE

This is to certify that the dissertation entitled **"Evaluation of Anti-arthritic activity of the ethanolic extract of Mimosa pudica Linn in Complete Freund's adjuvant induced arthritis in wistar rats"** submitted by **Register No: 261525003** in partial fulfillment for degree of **Master of Pharmacy in Pharmacology** in partial fulfillment of the course for the award of the degree of **Master of Pharmacy in Pharmacology.** It was carried out at Department of Pharmacology in C.L. Baid Metha College of Pharmacy, Chennai-97 under my guidance during the academic year 2016-2017.

Place: Chennai **Dr.P.AMUDHA.,M.Pharm.Ph.D.**

Date:

Phone : 24960151, 24962592, 24960425
e-mail : clbaidmethacollege@gmail.com
Website : www.clbaidmethacollege.com

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Prof.Dr.GRACE RATHNAM, M.Pharm., Ph.D.,
Principal

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Place: Chennai

Date: **Prof.Dr.GRACE RATHNAM., M.Pharm.Ph.D.,**

Phone : 24960151, 24962592, 24960425
e-mail : clbaidmethacollege@gmail.com
Website : www.clbaidmethacollege.com

C.L. Baid Metha College of Pharmacy

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Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai.
Approved by Pharmacy Council of India, New Delhi, and
All India Council for Technical Education, New Delhi

Dr.P.MURALIDHARAN, M.Pharm., Ph.D.,
Professor & Head
Department of Pharmacology,

CERTIFICATE

This is to certify that the dissertation entitled**"Evaluation of Anti-arthritic activity of the ethanolic extract of Mimosa pudica Linn in Complete Freund's adjuvant induced arthritis in wistar rats"**submitted by **Register No: 261525003** in partial fulfillment for degree of **Master of Pharmacy in Pharmacology** in partial fulfillment of the course for the award of the degree of **Master of Pharmacy in Pharmacology.** It was carried out at Department of Pharmacology in C.L. Baid Metha College of Pharmacy, Chennai-97 under my guidance during the academic year 2016-2017.

Date: **Dr.P.MURALIDHARAN.,M.Pharm., Ph.D.**

Place: Chennai

DECLARATION

Register No.261525003, hereby declare that this dissertation entitled,**"Evaluation of Anti-arthritic activity of the ethanolic extract of Mimosa pudica Linn in Complete Freund's adjuvant induced arthritis in wistar rats"** has been originally carried out by me under the guidance and supervision of Prof. Dr.P.Amudha, M.Pharm,. Ph.D., Asst Professor for the department of pharmacology, C.L. Baid Metha College of Pharmacy, Chennai-97 for the academic year 2016-2017. This work has not been submitted in any other degree at any other university.

Date: **Register No**. 261525003

Place: Chennai

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I. INTRODUCTION

1.1. *Rheumatoid arthritis*

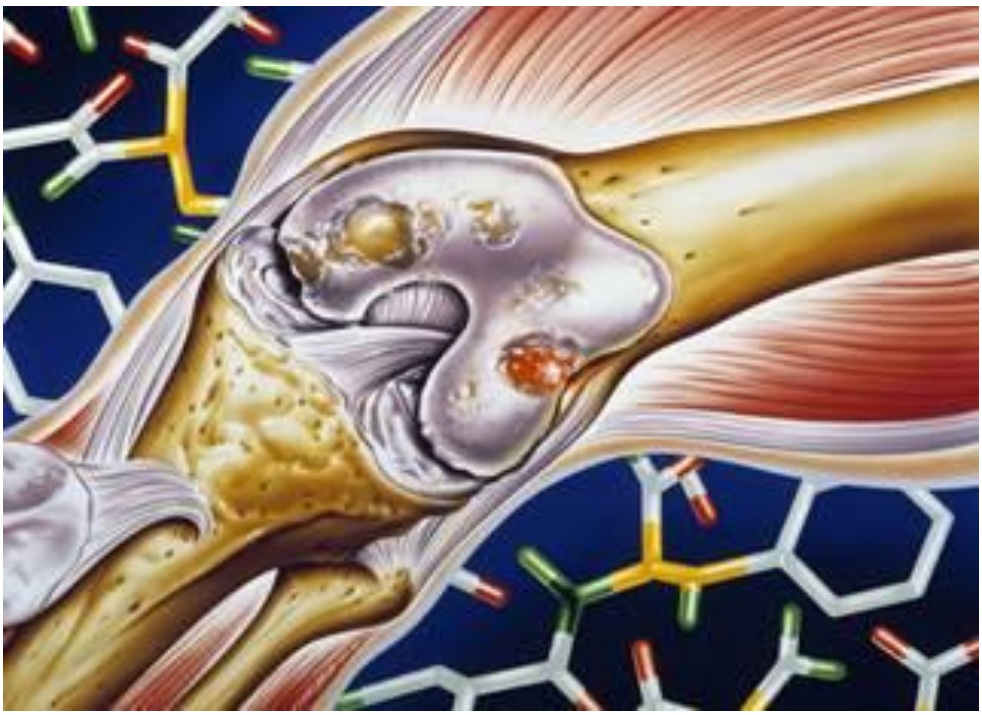


Figure A. Joint damage in rheumatoid arthritis

Rheumatoid arthritis is a chronic, systemic inflammatory disorder or a long term auto immune multisystem illness in which the body's immune system attacks the body's tissues and joints mistakenly causing an inflammatory synovitis which often progresses the destruction of joint ankylosis and articlular cartilage[1]. An autoimmune disease is a condition which arises from an abnormal response to our normal immune system. The immune system is a host defence mechanism comprising complex organisation of cells and antibodies designed normally to "seek and destroy" invaders of the body. The synovium(inside of joints) is a thin delicate lining serves as an important source of nutrients for cartilage which thickens during RA resulting in inflammation and pain in and around the joints. Additionally, synovial cells synthesize joint lubricants and helps them move smoothly such as collagens, as well as fibronectin and hyaluronic acid that constitute the structural framework of the synovial interstitium[2].

Rheumatoid arthritis is influenced by the following factors such as gender, age, environmental factors and reproductive status, various studies demonstrate that genetic factors also play a major role on an individual's susceptibility to RA. It is characterized by periods of disease flares and remissions. Chronic inflammation of rheumatoid arthritis can cause permanent joint destruction and deformity. It leads to warm, swollen, painful and stiff joints which gets worsened following rest. Usually multiple joints of the fingers and hands, wrists, feet and knees typically gets affected in a symmetrical distribution (affecting both sides of the body). It may also affect other parts of the body and this may result in a low red blood cell count, inflammation around the lungs, and inflammation around the heart[3].

1.2. History

The hallmark feature of rheumatoid arthritis (RA) is persistent symmetric polyarthritis (synovitis) that affects the hands and feet, although any joint lined by a synovial membrane may be involved. The severity of RA may fluctuate over time, but chronic RA most commonly results in the progressive development of various degrees of joint destruction, deformity, and a significant decline in functional status. Extra-articular involvement of organs such as the skin, heart, lungs, and eyes can also be significant.

Juvenile idiopathic arthritis (JIA), sometimes referred to as juvenile rheumatoid arthritis (JRA), is the most common form of childhood arthritis. In most patients, the immunogenic associations, clinical pattern, and functional outcome of JIA are different from those of adult-onset RA.

Patients with RA may report difficulty performing activities of daily living (ADLs), such as dressing, standing, walking, personal hygiene, or use of their hands. In addition to articular deterioration, constitutional symptoms (eg, fatigue, malaise, morning stiffness, weight loss, and low-grade fever) may be present.

In most patients, RA has an insidious onset. It may begin with systemic features (eg, fever, malaise, arthralgias, and weakness) before the appearance of overt joint inflammation and swelling. A small percentage (approximately 10%) of patients with this disease have an abrupt

onset with the acute development of synovitis and extra-articular manifestations. Spontaneous remission is uncommon, especially after the first 3-6 months[4].

1.3. Epidemiology

Worldwide, the annual incidence of RA is approximately 3 cases per 10, 000 population, and the prevalence rate is approximately 1%, increasing with age and peaking between the ages of 35 and 50 years. RA affects all populations, though it is much more prevalent in some groups (eg, 5-6% in some Native American groups) and much less prevalent in others (eg, black persons from the Caribbean region).

First-degree relatives of individuals with RA are at 2- to 3-fold higher risk for the disease. Disease concordance in monozygotic twins is approximately 15-20%, suggesting that nongenetic factors play an important role. Because the worldwide frequency of RA is relatively constant, a ubiquitous infectious agent has been postulated to play an etiologic role.

Women are affected by RA approximately 3 times more often than men, but sex differences diminish in older age groups[5, 6] .

In investigating whether the higher rate of RA among women could be linked to certain reproductive risk factors, a study from Denmark found that the rate of RA was higher in women who had given birth to just 1 child than in women who had delivered 2 or 3 offspring[7]. However, the rate was not increased in women who were nulliparous or who had a history of lost pregnancies[6].

Time elapsed since pregnancy is also significant. In the 1- to 5-year postpartum period, a decreased risk for RA has been recognized, even in those with higher-risk HLA markers. [8]

The Danish study also found a higher risk of RA among women with a history of preeclampsia, hyperemesis during pregnancy, or gestational hypertension. [6]

In the authors view, this portion of the data suggested that a reduced immune adaptability to pregnancy may exist in women who are predisposed to the development of RA or that there may be a link between fetal microchimerism (in which fetal cells are present in the maternal circulation) and RA. [6]

1.4 Classification of inflammatory arthritis

Inflammatory arthritis is generally classified into seropositive and seronegative groups[9]. These are based on the presence of rheumatoid factor, an immunoglobulin which reacts with gamma globulin, in the blood of the majority of patients with seropositive disease and in a small minority of patients with seronegative disease. The prototype seropositive form of arthritis is rheumatoid arthritis. Other members include the group of conditions labelled collagen vascular diseases, such as systemic lupus erythematosus, scleroderma, vasculitis, Sjogren's syndrome. Members of this group include ankylosing spondylitis, psoriatic arthritis, reactive arthritis, and arthritis of inflammatory bowel disease. In addition to the presence of rheumatoid factor, there are extra-articular features which distinguish the seropositive from the seronegative forms of inflammatory arthritis.

Seropositive is the most common diagnosis among rheumatoid arthritis patients. Being seropositive means that your blood tests show the presence of antibodies that can cause symptoms of rheumatoid arthritis. In most cases of rheumatoid arthritis diagnoses, the patient tests positive for rheumatoid factor and/or anti-CPP antibodies. These indicate that the patient is seropositive and that they possess the antibodies that cause an attack on joints and lead to inflammation.

Seronegative rheumatoid arthritis is the diagnosis of rheumatoid arthritis without the presence of certain antibodies in the patient's blood. It is one of two main types of rheumatoid arthritis diagnoses

Among the seronegative inflammatory joint diseases is a group labelled spondyloarthritis[10]. This condition is characterized by inflammatory disease of the joints of the back, both the sacroiliac joints and the apophyseal joints of the spine.

Seropositive rheumatoid arthritis is thought to present a more difficult and severe course of symptoms than seronegative patients. However, this isn't always the case and the treatment options available for seropositive patients can allow rheumatoid arthritis sufferers to still enjoy their quality of life.

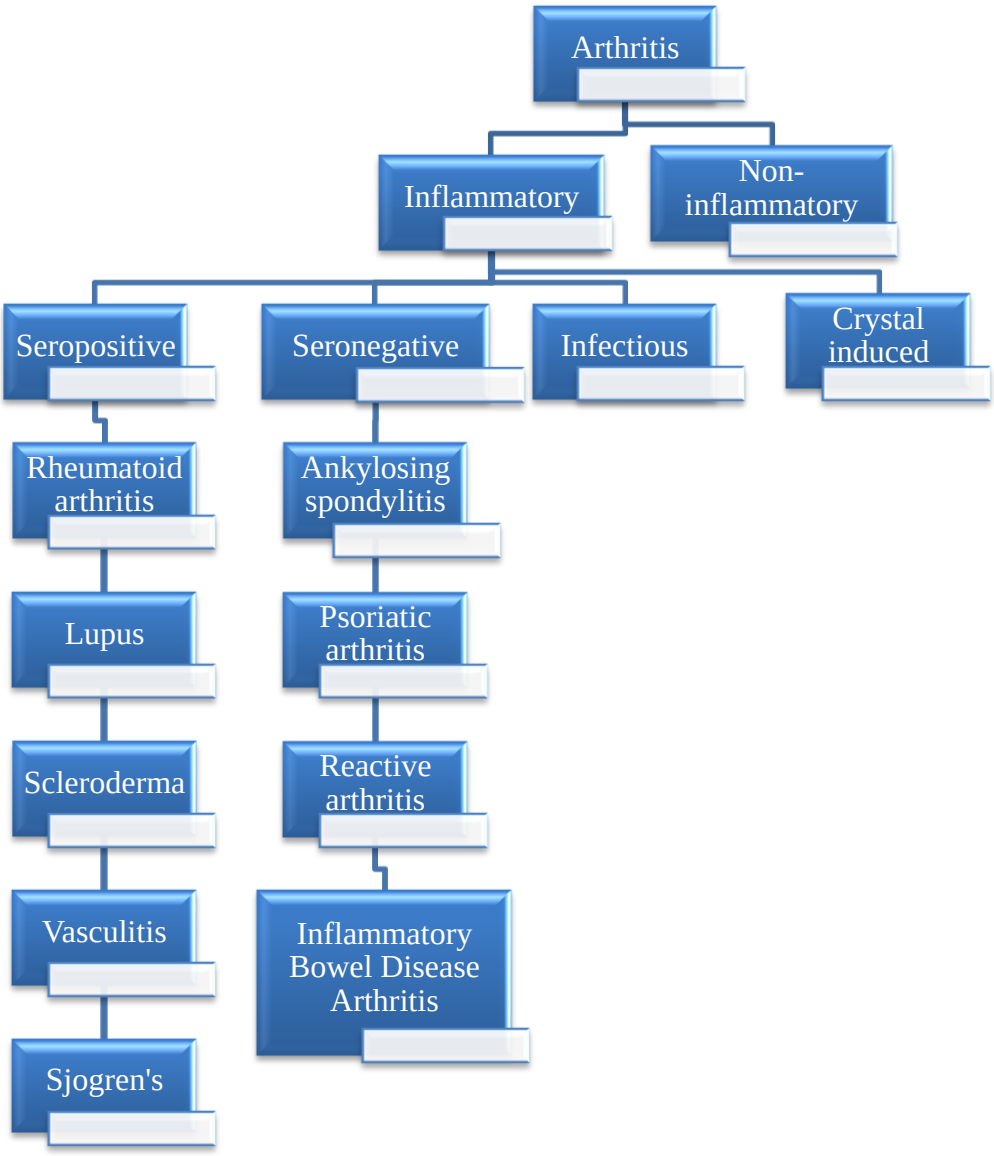


Figure B. Classisfication of rheumatoid arthritis

1.5 Different stages of Rheumatoid arthritis[11,12]

Three identified stages of rheumatoid arthritis, characterized by differing pathogenesis and physical changes:

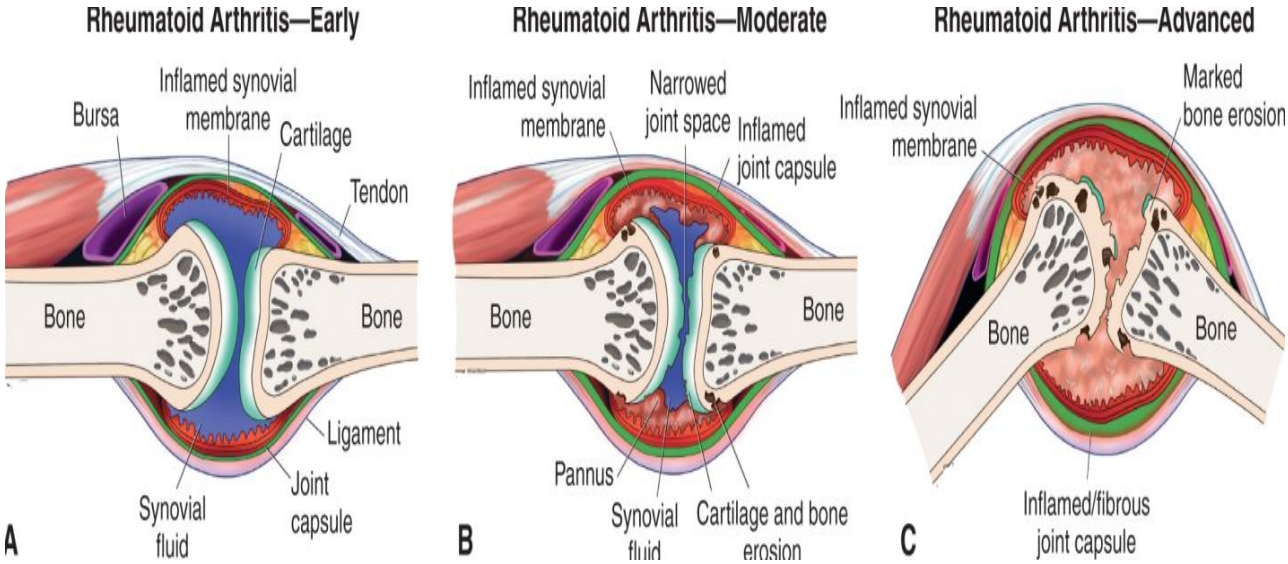
Stage I: Early stage RA is notable by the presence of synovial membrane inflammation, which results in joint swelling and pain on motion. Immune cells move to the inflammation site, leading to high cell counts in synovial fluid; however, x-rays are typically negative, other than showing the possible presence of some osteoporosis and soft tissue swelling. Treatment of early-stage RA focuses on joint protection and inflammation control.

Stage II: In moderate stage RA, there is T and B cell proliferation and angiogenesis in the synovium. Synovial tissue starts to grow into the joint cavity, across cartilage, which will be gradually destroyed. The joint begins to narrow because of cartilage loss. There are typically no joint deformities at this stage, though mobility may become limited with adjacent muscle atrophy. There may be mild malaise as well as the presence of nodules. Treatment goals are the same as stage I RA.

Stage III: End-stage or advanced RA disease results in a cessation of inflammatory processes. The formation of fibrous tissue and/or bone ankylosing (fusing of bone) results in ceased joint function. MRI will show proliferative pannus (a membrane of granulation tissue). Patient symptoms are much the same as in stage III, i.e. joint pain, swelling, stiffness, weakness andmalaise. At this point, treatment goals focus on reduction of pain and halting additional joint damage. Patients with end-stage RA may undergo joint replacement surgery.

RA progression:

How RA progresses and expresses itself varies widely from patient to patient. This variability can be seen in patterns of joint involvement, as well as the degree of disease involvement outside of the joints, including development of rheumatoid nodules, lung involvement, and other RA-related complications. One patient may experience RA symptoms



that affect only small joints, while another may have symptoms that affect only large joints. Still other patients will experience more generalized disease that affects a range of joints, regardless of size. There is also wide variation among patients in how active the disease is and how quickly structural damage in the joints develops. Progressive rheumatoid arthritis requires a deliberate treatment plan provided by a team of physicians and specialists. This plan should be tailored specifically to your individual symptoms and history of the disease.

Figure C. Stages of Rheumatoid arthritis

1.6 Signs and symptoms[13,14]

The onset of rheumatoid arthritis in most patients is insidious, often beginning with fever, malaise, arthralgias, and muscle weakness before progressing to inflammation and swelling of the joints.

RA typically manifests with the following:

- Persistent symmetric polyarthritis (synovitis) of hands and feet (hallmark feature)
- Progressive articular deterioration
- Extra-articular involvement
- Difficulty performing activities of daily living (ADLs)
- Constitutional symptoms

The physical examination should address the following:

- Upper extremities (metacarpophalangeal joints, wrists, elbows, shoulders)
- Lower extremities (ankles, feet, knees, hips)
- Cervical spine

During the physical examination, it is important to assess the following:

- Stiffness
- Tenderness
- Pain on motion
- Swelling
- Deformity
- Limitation of motion
- Extra-articular manifestations
- Rheumatoid nodules

1.7 Pathophysiology

The pathogenesis of RA is not completely understood. An external trigger (eg, cigarette smoking, infection, or trauma) that sets off an autoimmune reaction, leading to synovial hypertrophy and chronic joint inflammation along with the potential for extra-articular manifestations, is theorized to occur in genetically susceptible individuals[15].

Synovial cell hyperplasia and endothelial cell activation are early events in the pathologic process that progresses to uncontrolled inflammation and consequent cartilage and bone destruction. Genetic factors and immune system abnormalities contribute to disease propagation.

CD4 T cells, mononuclear phagocytes, fibroblasts, osteoclasts, and neutrophils play major cellular roles in the pathophysiology of RA, whereas B cells produce autoantibodies (ie, rheumatoid factors).

Abnormal production of numerous cytokines, chemokines, and other inflammatory mediators has been demonstrated in patients with RA, including the following[16]:

- Tumor necrosis factor alpha (TNF-α)
- Interleukin (IL)-1
- IL-6
- IL-8
- Transforming growth factor beta (TGF-ß)
- Fibroblast growth factor (FGF)
- Platelet-derived growth factor (PDGF)

Ultimately, inflammation and exuberant proliferation of the synovium (ie, pannus) leads to destruction of various tissues, including cartilage (see the image below), bone, tendons, ligaments, and blood vessels. Although the articular structures are the primary sites involved by RA, other tissues are also affected.

Findings from studies of gene–environment interactions complement these observations. Smoking and other forms of bronchial stress (e.g., exposure to silica) increase the risk of rheumatoid arthritis among persons with susceptibility HLA– DR4 alleles. Moreover, smoking and HLA-DRB1 alleles synergistically increase one’s risk of having ACPA.

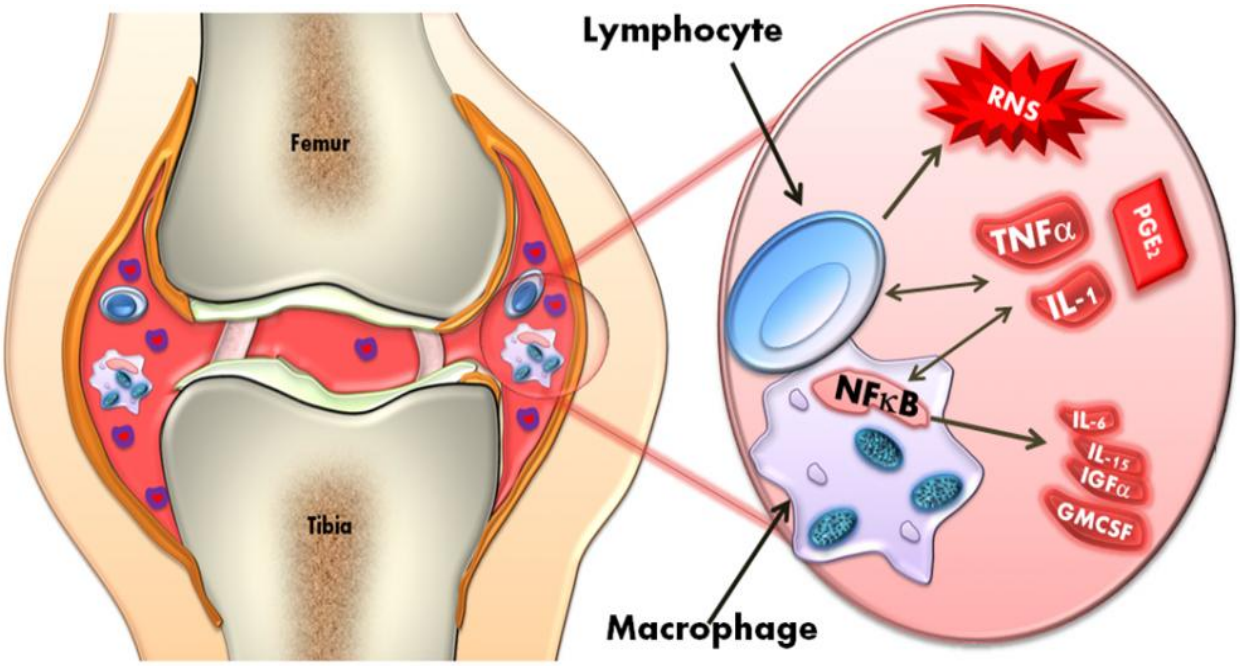


Figure D. Pathophysiology of RA

Disease Initiation

The search for an elusive single trigger for RA has been ongoing for many years. Multiple studies have failed to conclusively demonstrate that any organism or exposure is singly responsible for the disease. However, a number of well done epidemiological studies and genetic studies have provided valuable information to inform our genera, albeit still incomplete, understanding of the dynamic process of disease initiation.

Genetic Susceptibilities[17]

In the early 1980's an association was described for the association of RA with class II major histocompatability (MHC) antigens, specifically the shared epitope found in HLA-DR4. Class II MHC on the surface of an antigen presenting cell interacts with a T cell receptor in the context of a specific antigen, usually a small peptide sequence from a protein. A sequence of amino acid residues with highly conserved sequence and charge characteristics within the hypervariable region of HLA-DR4 remains the largest genetic risk factor described for RA, estimated to contribute approximately 30% of the genetic risk for the disease. It is hypothesized

that a triggering peptide (or peptides) with a tight conformational fit for the pocket formed by these residues is an early event leading to the activation of T lymphocytes. More recently, it has been found that modified citrullinated peptides may have significant binding specificity for shared epitope alleles, with some data now suggesting that citrullinated sequences from different proteins are associated with allelic restriction. (A more detailed discussion of citrullination is below).

Other genetic susceptibilities have been described in RA, but their relative contributions to the disease are still not well defined. These include peptidyl arginine deiminase-4 (PAD-4) which may lead to increased citrullination, PTNP22, STAT4, and CTLA4 which may be involved in T cell activation, TNF receptors, and others.

That RA has a genetic component is also borne out through a number of studies of monozygotic (from the same embryo, thus nearly identical DNA) and dizygotic (from different embryos) twins. In these studies the concordance rates between twins was higher in monozygotic twins ranging from 15-35% compared with dizygotic twins in which the concordance was in the 5% range. Even the dizygotic RA prevalence was higher than the general population estimates of approximately 1%. It is important to emphasize however that even in twins with nearly identical DNA, there was far from perfect correlation of the development of RA, implicating many other factors related to the development of disease than genetic factors.

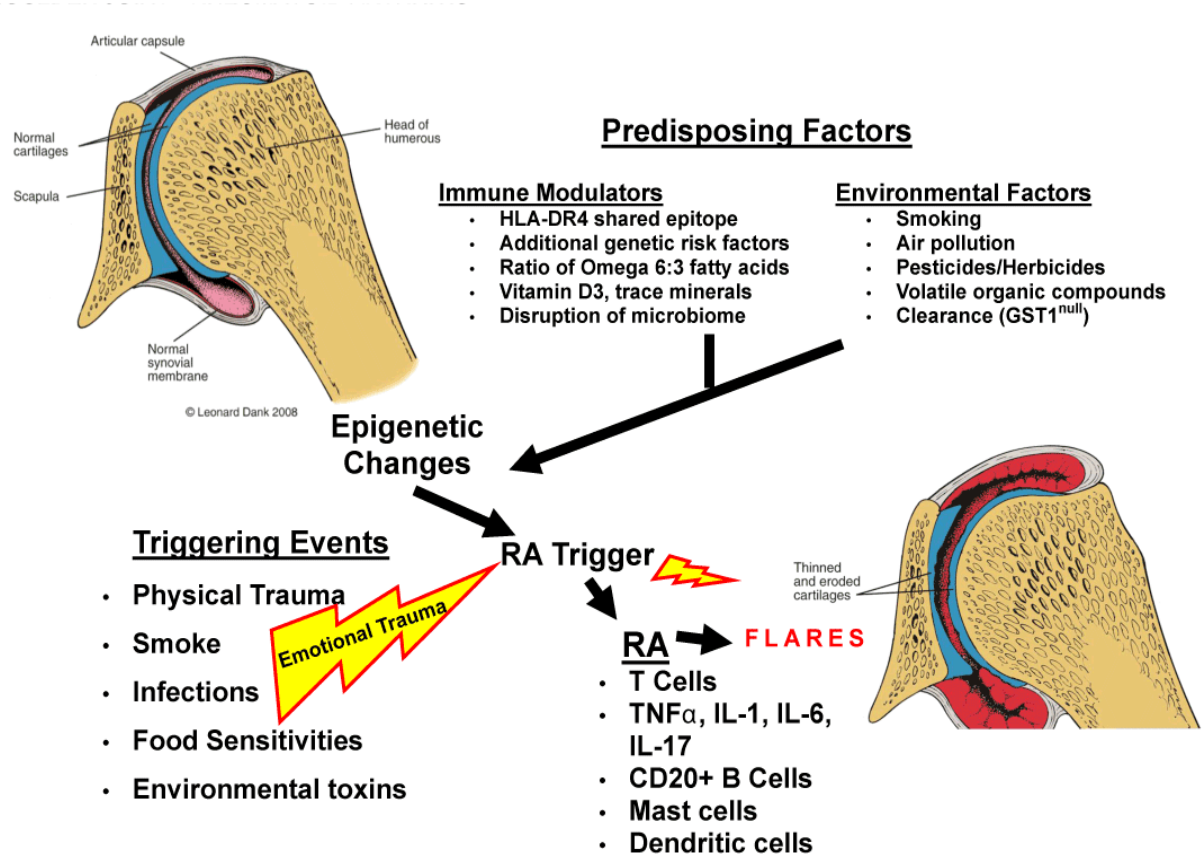


Figure E. Risk factors of RA

Triggers of Disease

The fact that there is not perfect genetic concordance implicates other factors in disease development. A search for these elusive triggers has been largely unrevealing. A number of well performed studies have demonstrated that cigarette smoking is a significant risk factor for the development of disease and also with disease severity. Interestingly this relationship is especially strong in individuals who carry the shared epitope, and even more in patients who have RA auto antibodies.

The search for bacterial or viral infections as causes of RA have often been hypothesized, and many patients will relate the onset of their symptoms to an antecedent infection; however, the recovery of organisms or their DNA from blood or joint tissue have been unfruitful in discovering "the" elusive infection responsible for RA. Nonetheless, the ability of an infection to

activate a number of immunological and inflammatory pathways may “prime the pump” in combination with other factors.

Perhaps the most exciting developments in the last few years in terms of RA initiation has been the growing research to evaluate the possible role of oral bacteria as a trigger for RA. There has been a longstanding association described between periodontal disease with RA, however cause and effect has been far from proven. Periodontal disease is characterized by significant inflammation of the gums that leads to bone destruction and collagen matrix destruction. Both are inflammatory diseases with many of the same mediators and pathways involved, thus this could simply be an association between two inflammatory processes. However, it is now recognized that a specific species of bacteria, *Porphyromonas gingivalis*, which colonizes patients with periodontal disease and marks the progression from gingivitis to more aggressive periodontitis has an enzyme that can cause citrullination of proteins. With the growing recognition that protein citrullination is an early event leading to an immune response against these in RA, these data suggest that periodontal infection may precede the development of RA in some patients serving as a disease initiation factor. A number of groups worldwide, including our own, are now investigating these pathways to better understand these processes.

Citrullination

The recognition of antibodies directed against citrullinated peptides in RA has been a major development to improve disease identification and provide prognostic information. Citrulline is a post-translational modification that occurs on arginine residues contained within proteins and peptides. There are a number of enzymes that can cause citrullination to occur, present in various cell types and tissues known as peptidylarginine deiminases (PADs). Citrullination is a normal process, required for normal skin formation and other physiologic functions. However, in rheumatoid arthritis an autoimmune response develops against citrullinated peptides detected as anti-citrullinated peptide antibodies (ACPA). One of tests to detect these antibodies detects anti-cyclic citrullinated peptides (anti-CCP), currently the most commonly used diagnostic test for them. The presence of anti-CCP are >98% specific for the diagnosis of rheumatoid arthritis; however, not all patients with RA will develop anti-CCP antibodies[18].

Of significant importance is the recognition that these anti-CCP antibodies may be detected up to 15 years before the onset of clinical symptoms of RA indicating a preclinical phase of disease in which immunologic activation is already ongoing[19]. Moreover, it has recently been demonstrated that specific citrullinated peptide sequences bind to shared epitope alleles with high affinity and can lead to T cell activation.

The mechanisms to citrullination that lead to RA remain unclear. A polymorphism in the PAD4 gene which may lead to increased citrullination has been described populations. In RA patients, autoantibody responses also develop against the PAD4 protein, associated with a more aggressive disease course. One species of oral bacteria *Porphyromonas gingivalis* has a PAD enzyme. Given the relationships described with periodontal disease and RA, it has been hypothesized that this bacteria may also serve to initiate citrullination in the preclinical phases of RA.

1.8 Propagation of Disease

T cell activation

Upon encounter with antigen in the context of MHC on an antigen presenting cell, a T-lymphocyte is positioned for 3 possible fates: activation, anergy / tolerance, or apoptosis (death). T cell activation is only possible if the T cell receives a "second signal" through engaging additional cellular receptors. One of the most important of these second signals is delivered through the CD28 molecule on the surface of the T cell but many other second signals are involved in this process of "costimulation"[20]. Upon engagement of these receptors, a T cell usually becomes activated. Failure to engage the stimulatory receptors, or engagement of a down-regulator receptor will cause the cell to become tolerant to the antigen (eg does not activate when exposed to the antigen) or to undergo programmed cell death through apoptosis. The process of T-cell costimulation is interrupted by abatacept, a biological therapy used to treat RA.

When T cells become activated, they will in turn proliferate and begin to secrete additional cytokines including IL-2 which furthers their proliferation, and depending on other exposures, cytokines such as IFN-γ, TNF, and IL-4[21]. It is the effect of these T-cell derived

cytokines that additional cells become activated. T cells also directly interact through surface receptors with other cells to generate additional activation signals.

B Cell Activation and Autoantibodies

B cells become activated through interactions with T cells and through soluble cytokines that enhance their proliferation and differentiation. B cells express a number of receptors on their surfaces during their differentiation, including the molecule CD20, which is lost upon terminal differentiation to antibody-forming plasma cells. B cells and plasma cells can be found in rheumatoid synovium sometimes as lymphoid aggregates in the subsynovium. The effects of B cells extend beyond their roles in forming plasma cells including cytokine production, direct cellular interactions, and they themselves serve as antigen-presenting cells to T lymphocytes. The role of B cells in RA has been clearly demonstrated with the efficacy of rituximab which eliminates circulating B cells, though with limited impact on autoantibody formation.

One of the features of most autoimmune diseases is the presence of disease-specific autoantibodies that help to define disease phenotypes. Antibodies are made by plasma cells, which represent the terminal stage of differentiation for B lymphocytes[22]. Rheumatoid arthritis is characterized by the presence of autoantibodies known as rheumatoid factors (R_f) and anti-citrullinated peptide antibodies (ACPA, which includes the anti-cyclic citrullinated peptide antibody or anti-CCP). Rheumatoid factors have been long recognized as a feature of many patients with RA. These are autoantibodies in the classical sense; they are antibodies directed against native antibodies, most classically described as IgM antibodies that recognize the Fc portion of IgG molecules, but RF may also be of the IgG or IgA isotypes. Rheumatoid factors are not specific for the diagnosis of RA, but are seen in many other inflammatory and autoimmune conditions. These include Sjogren's syndrome, chronic infections including tuberculosis and endocarditis, hepatitis C, chronic kidney or liver disease, lymphoproliferative diseases including myeloma, and other conditions. While the rheumatoid factor may be seen in other inflammatory conditions, ACPA are highly specific for rheumatoid arthritis and define a more aggressive disease phenotype .

Effector Cell Activation

While T cells and B cells represent the immunological aspects of RA, most of the damage from the disease is driven through effector cells and their products including cytokines and other mediators. The synovial lining in RA represents an expansion of fibroblast like cells and macrophages. It is the macrophage that has been seen as one of the master orchestrators of the effector damage in RA. Macrophages are rich sources and major producers of proinflammatory cytokines including TNF, IL-1, IL-6, IL-8, and GM-CSF[23]. These cytokines further stimulate the macrophage, as well as other cells in the microenvironment in a including fibroblasts and osteoclasts, and finally at distant sites in the body through cell surface receptors including the hepatocyte which is responsible for the generation of acute phase response proteins (such as C-reactive protein). Macrophages are also producers of prostaglandins and leukotrienes, nitric oxide, and other pro-inflammatory mediators with local and systemic effects. The **synovial fibroblast**, also secretes cytokines including IL-6, IL-8 and GM-CSF, and other mediators including destructive proteases and collagenases.

Neutrophils are recruited in very large numbers to the rheumatoid cavity where they can be aspirated in the synovial fluid. The recruitment of neutrophils to the joint is likely driven by IL-8, leukotriene B4, and possibly localized complement activation through C5a. Neutrophils in the synovial fluid are in an activated state, releasing oxygen-derived free radicals that depolymerize hyaluronic acid and inactivate endogenous inhibitors of proteases, thus promoting damage to the joint.

Chondrocytes, like synovial fibroblasts, are activated by IL1 and TNF to secrete proteolytic enzymes. They may, therefore, contribute to the dissolution of their own cartilage matrix, thus explaining the progressive narrowing of joint spaces seen radiographically in this disease[24].

1.9 Inflammatory Mediators in RA

Cytokines

One of the most important group of mediators in RA are cytokines. The most prominent of these are **TNF, IL-1, and IL-6**. These cytokines, released in the synovial microenvironment have autocrine (activating the same cell), paracrine (activating nearby cells), and endocrine (acting at distant sites) effects and accounting for many systemic manifestations of disease. There are many shared functions of TNF, IL-1, and IL-6, and these cytokines in turn upregulate the expression of the others. Among the important effects of these cytokines are[25]:

- Induction of cytokine synthesis
- Upregulation of adhesion molecules
- Activation of osteoclasts
- Induction of other inflammatory mediators including prostaglandins, nitric oxide, mtrix metalloproteinases
- Induction of the acute phase response (e.g. C-reactive protein, increased ESR)
- Systemic features (e.g., fatigue, fever, cachexia)
- Activation of B cells (IL-6)
- Other cytokines are increasingly described in RA. These include **IL-8** which is involved in cellular recruitment, **GM-CSF** involved in macrophage development, **IL-15** involved in T cell proliferation, **IL-17** which has pleiotropic effects on multiple cell types including osteoblast expression of RANK leading to osteoclast activation, and **IL-23** involved in increasing TH17 cell differentiation.
- Soluble mediators of inflammation that may diffuse in from blood and/or be formed locally within the joint cavity includes **prostaglandins, leukotrienes, matrix and metalloproteinases**. Prostaglandins are involved in pain senstitization localized inflammation, and some effects on bone,and leukotrienes play roles in vascular permeability and chemotaxis. Matrix metalloproteinases (MMPs) are potent in their ability to enzymatically degrade the collagen matrix of cartilage. Kinins cause release of prostaglandins from synovial fibroblasts, and are also potent algesic (pain-producing) agents. Complement may be available for interaction with immune complexes to generate additional chemotactic stimuli. The neuropeptide substance P is a potent vasoactive, proinflammatory peptide that has also been implicated in RA.

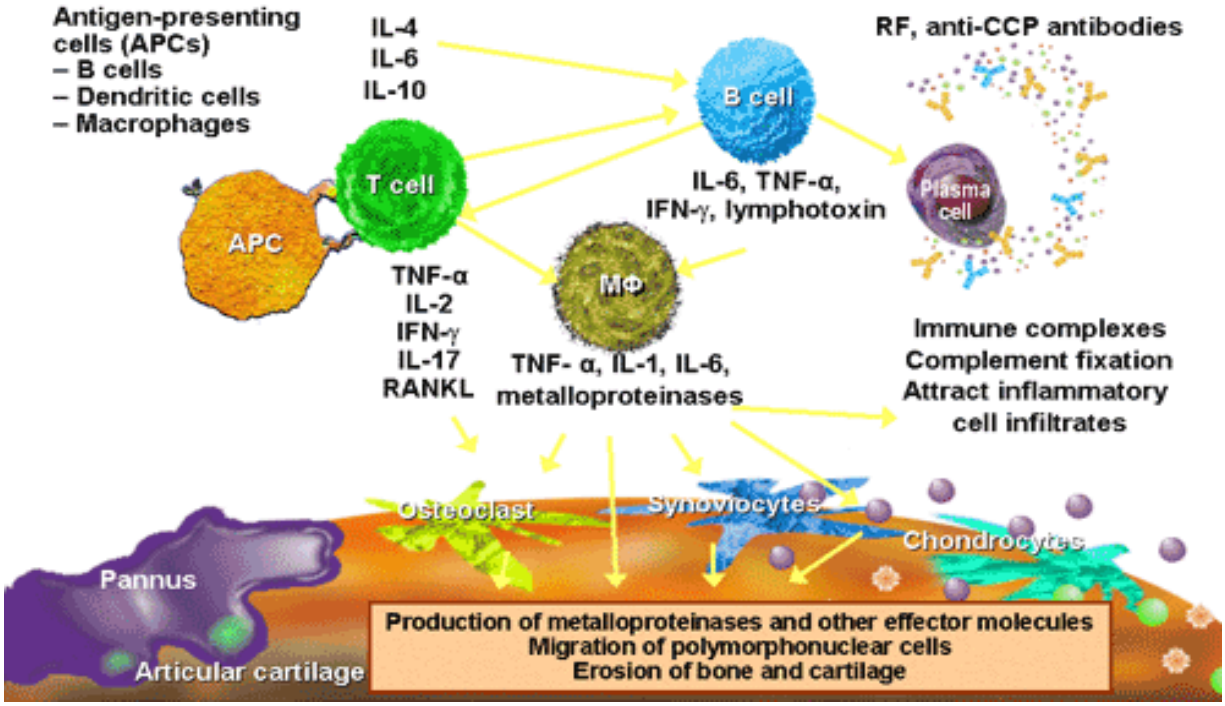


Figure F. Role of inflammatory mediators in RA

1.10 Diagnosis[26, 27, 28]

No test results are pathognomonic; instead, the diagnosis is made by using a combination of clinical, laboratory, and imaging features. Potentially useful laboratory studies in suspected RA include the following:

- Erythrocyte sedimentation rate
- C-reactive protein level
- Complete blood count

- Rheumatoid factor assay
- Antinuclear antibody assay
- Anti−cyclic citrullinated peptide and anti−mutated citrullinated vimentin assays

Potentially useful imaging modalities include the following:

- Radiography (first choice): Hands, wrists, knees, feet, elbows, shoulders, hips, cervical spine, and other joints as indicated
- Magnetic resonance imaging: Primarily cervical spine
- Ultrasonography of joints: Joints, as well as tendon sheaths, changes and degree of vascularization of the synovial membrane, and even erosions

Joint aspiration and analysis of synovial fluid may be considered, including the following:

- Gram stain
- Cell count
- Culture
- Assessment of overall appearance

1.11 Management of RA[29, 30]

Nonpharmacologic, nonsurgical therapies include the following:

- Heat and cold therapies
- Orthotics and splints
- Therapeutic exercise
- Occupational therapy
- Adaptive equipment
- Joint-protection education
- Energy-conservation education

Nonbiologic disease-modifying antirheumatic drugs (DMARDS) include the following:

- Hydroxychloroquine
- Azathioprine
- Sulfasalazine
- Methotrexate

- Leflunomide
- Cyclosporine
- Gold salts
- D-penicillamine
- Minocycline

Biologic tumor necrosis factor (TNF)–inhibiting DMARDs include the following:

- Etanercept
- Infliximab
- Adalimumab
- Certolizumab
- Golimumab

Biologic non-TNF DMARDs include the following:

- Rituximab
- Anakinra
- Abatacept
- Tocilizumab
- Sarlilumab
- Tofacitinib

Other drugs used therapeutically include the following:

- Corticosteroids
- Nonsteroidal anti-inflammatory drugs (NSAIDs)
- Analgesics

Surgical treatments include the following:

- Synovectomy
- Tenosynovectomy
- Tendon realignment
- Reconstructive surgery or arthroplasty

- Arthrodesis

1.12 Modes of action of Freund's adjuvant

Freund's adjuvants are irreplaceable components of induction protocols of many experimental animal models of autoimmune disease. Apart from the early studies done in the 1950s and 1960s, no further direct investigation on the mode of action of these adjuvants has been undertaken. It is generally assumed that incomplete (IFA) and complete Freund's adjuvant (CFA) act by prolonging the lifetime of injected autoantigen, by stimulating its effective delivery to the immune system and by providing a complex set of signals to the innate compartment of the immune system, resulting in altered leukocyte proliferation and differentiation. There are many evidences collected from various types of studies that provide more insight in the specific alterations of the immune response caused by IFA and CFA. Early events include rapid uptake of adjuvant components by dendritic cells, enhanced phagocytosis, secretion of cytokines by mononuclear phagocytes, and transient activation and proliferation of CD41 lymphocytes. The mycobacterial components within CFA signal T lymphocytes to assume a Th1 profile so that strong delayed-type hypersensitivity against autoantigens develops. In the absence of mycobacteria, T-lymphocyte differentiation tends to assume a Th2 pro- file with strong antibody production only. The mycobacterial component also accounts for a morphologic and functional remodeling of the haemopoietic system that develops over a period of several weeks and that is characterized by a drastic expansion of Mac-11 immature myeloid cells. These cells have been found to be associated with enhanced disease in some models but with reduced disease in others. Thus, in experimental autoimmune diseases, CFAmediated activation of the innate immune compartment is important not only by regulating the early induction phase but also by providing a surplus of effector and regulator cells in the late phase.

1.13 Why Methotrexate[31, 32, 33]?

Methotrexate is one of the most effective and widely used medications for treating rheumatoid arthritis (RA) and other inflammatory types of arthritis. It's also one of the safest arthritis drugs, insist rheumatologists, despite a common misconception among many patients and even some primary care physicians that methotrexate is highly toxic.

Confusion about this important medication's safety profile seems to exist because it is also used – in much higher doses – for treating some forms of cancer. Most patients who use methotrexate to treat their inflammatory arthritis take between 10 and 25 milligrams (mg) per week. By contrast, the doses used to treat leukemia and certain other types of cancer may be hundreds of times larger.

That's not to suggest that taking methotrexate is risk-free. A 2009 review of 21 studies found that 73 percent of RA patients who used the medication experienced at least one side effect. Yet the study indicates that most of these problems were relatively minor. What's more, doctors who prescribe methotrexate for arthritis say that following a few simple steps can make this drug even safer to use.

Folic Acid Is a Must

Understanding how methotrexate works helps explain why it can cause unwanted effects. Researchers originally developed methotrexate in the 1940s as a cancer drug. It stops malignant (or cancerous) cells from rapidly multiplying and spreading by blocking their access to folate, a form of vitamin B, which these cells need to survive.

Unfortunately, depleting the body of folate can affect healthy cells, too, especially those in the gastrointestinal (GI) tract, mouth, hair follicles and liver.

GI problems such as nausea and vomiting are the most common side effects associated with methotrexate, affecting between 20and 65 percent of RA patients who take the drug. While hair loss is a relatively uncommon side effect in patients who take methotrexate at such doses, up to one third develop mouth ulcers, or sores. Many also complain of headaches, fatigue and an overall "blah" feeling – sometimes called "methotrexate fog" – that can occur a day after receiving a dose of methotrexate (which is taken in pill form or injected once a week).

The good news: These side effects can often be short-circuited by taking a folic acid supplement. Folic acid is the synthetic form of folate. One study found that RA patients on methotrexate who took folic acid supplements lowered the risk of GI problems and mouth sores by 79 percent.

A few additional steps may help prevent or relieve GI and oral problems:

- **Split the dose**. Most arthritis patients take methotrexate orally, in a dose consisting of several pills. Some find that splitting the dose eases GI side effects; take half the pills in the morning and the other half 12 hours later, preferably with food.
- **Ask about medication**. For very severe stomach queasiness, your doctor can prescribe an anti-nausea drug such as ondansetron (*Zofran*).
- **Swap your pills**. When nothing else helps, switching from oral methotrexate to the injectable version can eliminate GI distress.
- **Try a rinse**. To relieve painful mouth sores, a salt-water rinse or special mouthwash containing lidocaine (a pain reliever) may help.

Protecting the Liver

Since methotrexate blocks folate, taking folic acid – the manmade version of the vitamin – might seem like it would be counterproductive. However, methotrexate appears to relieve pain and other RA symptoms through actions that are largely unrelated to folate. Investigators discovered that methotrexate causes cells to release a molecule called adenosine, which blocks other chemicals that promote inflammation.

Fighting inflammation helps relieve painful, swollen joints. But it is also noted that adenosine causes fibrosis, or buildup of scar tissue, in the liver; over time, that could result in liver disease. It is important to note that alcohol also releases adenosine in the liver. In rare cases, methotrexate users may develop fibrosis and inflammation in the lungs, though this is unlikely to be related to adenosine release.

Regular blood tests are also necessary to detect signs of other problems that can arise in methotrexate users, including a drop in white blood cells, which normally guard against infections. Also, some people experience a dip in production of blood platelets, which could cause abnormal bleeding. However, these changes in the blood often go away if you stop taking the drug temporarily (which should only be done under a doctor's supervision).

1.14 Etiology

The cause of RA is unknown. Genetic, environmental, hormonal, immunologic, and infectious factors may play significant roles. Socioeconomic, psychological, and lifestyle factors

(eg, tobacco use, the main environmental risk [34]) may influence disease development and outcome.

Genetic factors

Genetic factors account for 50% of the risk for developing RA. About 60% of RA patients in the United States carry a shared epitope of the human leukocyte antigen (HLA)-DR4 cluster, which constitutes one of the peptide-binding sites of certain HLA-DR molecules associated with RA (eg, HLA-DR beta *0401, 0404, or 0405). HLA-DR1 (HLA-DR beta *0101) also carries this shared epitope and confers risk, particularly in certain southern European areas. Other HLA-DR4 molecules (eg, HLA-DR beta *0402) lack this epitope and do not confer this risk.

Genes other than those of the major histocompatibility complex (MHC) are also involved. Results from sequencing genes of families with RA suggest the presence of several resistance and susceptibility genes, including *PTPN22* and *TRAF5*. [35, 36]

Juvenile idiopathic arthritis (JIA), also known as juvenile rheumatoid arthritis (JRA), is a heterogeneous group of diseases that differs markedly from adult RA.

JIA is known to have genetically complex traits in which multiple genes are important for disease onset and manifestations, and it is characterized by arthritis that begins before the age of 16 years, persists for more than 6 weeks, and is of unknown origin.[37] The *IL2RA/CD25* gene has been implicated as a JIA susceptibility locus, as has the *VTCN1* gene.[38]

Some investigators suggest that the future of treatment and understanding of RA may be based on imprinting and epigenetics. **RA is significantly more prevalent in women than in men**,[39,40] which suggests that genomic imprinting from parents participates in its expression.[41,42] Imprinting is characterized by differential methylation of chromosomes by the parent of origin, resulting in differential expression of maternal over paternal genes. [43]

Epigenetics is the change in DNA expression that is due to environmentally induced methylation and not to a change in DNA structure. Clearly, the research focus will be on environmental factors in combination with immune genetics.

Infectious agents

For many decades, numerous infectious agents have been suggested as potential causes of RA, including *Mycoplasma* organisms, Epstein-Barr virus (EBV), and rubella virus. This suggestion is indirectly supported by the following evidence:

- Occasional reports of flulike disorders preceding the start of arthritis
- The inducibility of arthritis in experimental animals with different bacteria or bacterial products (eg, streptococcal cell walls)
- The presence of bacterial products, including bacterial RNA, in patients' joints
- The disease-modifying activity of several agents that have antimicrobial effects (eg, gold salts, antimalarial agents, minocycline)

Emerging evidence also points to an association between RA and periodontopathic bacteria. For example, the synovial fluid of RA patients has been found to contain high levels of antibodies to anaerobic bacteria that commonly cause periodontal infection, including *Porphyromonas gingivalis*.[44,45]

Hormonal factors

Sex hormones may play a role in RA, as evidenced by the disproportionate number of females with this disease, its amelioration during pregnancy, its recurrence in the early postpartum period, and its reduced incidence in women using oral contraceptives. Hyper prolactinemia may be a risk factor for RA.[46]

Immunologic factors

All of the major immunologic elements play fundamental roles in initiating, propagating, and maintaining the autoimmune process of RA. The exact orchestration of the cellular and cytokine events that lead to pathologic consequences (eg, synovial proliferation and subsequent joint destruction) is complex, involving T and B cells, antigen-presenting cells (eg, B cells, macrophages, and dendritic cells), and various cytokines. Aberrant production and regulation of both proinflammatory and anti-inflammatory cytokines and cytokine pathways are found in RA.

T cells are assumed to play a pivotal role in the initiation of RA, and the key player in this respect is assumed to be the T helper 1 (Th1) CD4 cells. (Th1 cells produce IL-2 and interferon [IFN] gamma.) These cells may subsequently activate macrophages and other cell populations, including synovial fibroblasts. Macrophages and synovial fibroblasts are the main producers of TNF-a and IL-1. Experimental models suggest that synovial macrophages and fibroblasts may become autonomous and thus lose responsiveness to T-cell activities in the course of RA.

B cells are important in the pathologic process and may serve as antigen-presenting cells. B cells also produce numerous autoantibodies (eg, RF and ACPA) and secrete cytokines.

The hyperactive and hyperplastic synovial membrane is ultimately transformed into pannus tissue and invades cartilage and bone, with the latter being degraded by activated osteoclasts. The major difference between RA and other forms of inflammatory arthritis, such as psoriatic arthritis, lies not in their respective cytokine patterns but, rather, in the highly destructive potential of the RA synovial membrane and in the local and systemic autoimmunity.

Whether these 2 events are linked is unclear; however, the autoimmune response conceivably leads to the formation of immune complexes that activate the inflammatory process to a much higher degree than normal. This theory is supported by the much worse prognosis of RA among patients with positive RF results.

1.15 Articular and systemic effects of RA

Articular Manifestations

Inflammation and subsequent destruction of synovial joints is the hallmark of RA. Why the immune system is lured to attack and destroy still remains unknown, but great strides have been made in understanding how. Inflammation of the synovial tissue involves interactions between macrophages, T and B lymphocytes, synovial fibroblasts, and other cells of the inflamed synovium such as mast cells, dendritic cells and plasma cells. Neutrophils are rare in RA synovial tissue but abundant in RA synovial fluid. These cell-cell interactions occur both through

direct cell-cell contact, as well as through the effects of secreted mediators. Proinflammatory cytokines, such as TNFα, IL-1 and IL-6, orchestrate synovial inflammation and stimulate cartilage degradation. This occurs through formation of a distinct tissue termed synovial pannus which invades cartilage with the assistance of proteolytic enzymes. Concurrently osteoclasts, which can form within the pannus through fusion of monocytic precursors, invade bone and cause periarticular erosions.

RA can involve most synovial joints, but rarely the DIPs or the thoracic, lumbar and sacral spine. The most commonly affected joints include the MCP and PIP joints of the hands, wrists and MTP joints of the feet. Joint destruction begins early in the disease with erosive changes often seen after only six months. The clinical exam can disclose synovial thickening and swelling, indicators of joint inflammation. At the time of presentation, nearly 70% of radiographs can be normal, but MRI and ultrasound with power Doppler have higher sensitivity to detect smaller erosions and synovial inflammation, and may reveal changes even when X-rays are normal[47]. If RA is left untreated, progression to joint destruction, subluxation and severe disability are the likely outcomes.

Inflammation of tendon sheaths also contributes to RA pathology. Tenosynovitis of the flexor tendons can lead to trigger finger, and weakening of the extensor tendons of the hands from chronic inflammation can lead to tendon ruptures. Damage to supporting and tracking structures associated with tendons of the hand contribute to the formation of boutonniere and swan-neck deformities. Carpal tunnel syndrome secondary to median nerve compression by surrounding inflammation is also a common complication in RA patients.

Bone Manifestations[48]

The bones of RA patients are affected in both a local and systemic manner. At a local level, factors that stimulate osteoclasts resulting in increased bone resorption are released from inflammatory and fibroblastic pannus cells. Additionally, inflammatory cytokines prevent a compensatory increase in the rate of periarticular bone formation, resulting in net bone loss. This inhibition of osteoblastic activity is via a combination of impaired mineralization and impaired osteoblast differentiation. These processes combine to result in both periarticular osteopenia, one of the first radiographic signs of RA, and periarticular erosions, the hallmark of RA joint

destruction. The use of disease modifying agents to induce clinical remission allows for restoration of normal function of osteoclasts and osteoblasts and may result in repair of erosive damage.

Bony changes in RA patients are not only seen in a periarticular distribution. RA is a known risk factor for osteoporosis, with up to 30% of patients affected by some estimates[49]. Most studies agree that, unlike postmenopausal osteoporosis, the risk of osteoporosis in RA patients is greater at the femoral neck than in the spine, but both areas can be involved. Disease duration and severity, sex, body mass and the use of corticosteroids all influence the risk of osteoporosis in RA patients.

An additional consideration is that many RA patients are on bisphosphonate therapy for osteoporosis or prevention of glucocorticoid-mediated bone loss. Research into the impact of bisphosphonate use in patients undergoing surgical procedures is ongoing. Osteonecrosis of the jaw in patients on bisphosphonates undergoing dental surgery has been a specific concern, but the consequences of manipulation of the peripheral skeleton in patients on these medications are still incompletely understood. Most research into this topic is in animal systems, and there are suggestions that while bone healing is not prevented, there are differences in bone quality after bisphosphonate exposure.

Airway Manifestations[50]

The presence of airway disease in RA is estimated to affect 20–30% of patients. Manifestations can include cricoarytenoid arthritis, pulmonary fibrosis and small airway disease, typically seen as bronchiolitis obliterans on histopathology, with obstructive abnormalities on lung function testing. Lung disease is more frequent in RA patients who are male, seropositive, smoke, and have longstanding disease. Some types of RA-associated lung disease are steroid responsive, but some patients have a progressive course leading to end-stage fibrosis and death[3]. In addition to lung disease secondary to RA, patients are also at risk for pulmonary toxicities from RA-related medications, including methotrexate, leflunomide and even anti-TNF medications.

Cardiovascular Manifestations

RA patients have a 40% increased risk of mortality as compared to the general population after 20 years of disease. This increased risk of mortality is primarily attributed to an increased incidence of cardiovascular disease. A recent cohort study has suggested that the risk of cardiovascular events in RA patients is 2-fold higher than the general population, equivalent to the risk of patients with diabetes. The propensity for vascular changes is found even in newly diagnosed patients, indicating that common mechanisms may exist linking synovitis resulting in joint destruction with endothelial dysfunction resulting in atherosclerosis.

The risk of cardiovascular disease and death increases with more severe disease and elevated inflammatory markers. Despite improved treatments for the symptoms of RA, the mortality risk has not improved over the past two decades. Whether this continued risk of death reflects an inability to control cardiovascular risk factors with immunomodulatory treatment or a lack of long-term follow up in patients treated with newer medications remains to be determined. Additionally, the optimal management of traditional cardiovascular risk factors, such as elevated cholesterol, has not been determined for RA.

1.16 COMPLICATIONS OF MEDICAL TREATMENT OF RA

[51,52]RA itself confers an elevated risk of infection, and DMARD and biologic therapies suppress the immune system through various targets, also increasing this risk. Bacterial infections, particularly pneumonia and soft-tissue infections,are increased with the use of methotrexate, and this is increased 2–4-fold with the addition of an anti-TNF medication. Similar infectious risks have been found with other biologic DMARDs as well. A significant risk of reactivation of tuberculosis has also been noted with anti-TNF medication. Thus, screening for TB exposure and treatment of latent TB prior to initiation of anti-TNF agents is recommended. Similar precautions are in place for other RA biologics, although the actual risk of TB due to these medications is less well understood.

TNF blockers also increase the risk for severe and systemic fungal infections such as histoplasmosis and coccidioidomycosis, which may be a significant issue in specific geographic locales.

An increased risk of viral infections with traditional or biologic DMARDs, including varicella-zoster virus, Epstein-Barr virus and cytomegalovirus has been documented.

Hepatitis B and C reactivation have also occurred with biologic DMARDs, so screening prior to treatment and vaccination when possible is recommended. Progressive multifocal leukoencephalopathy, an infection caused by reactivation of the JC virus, has also been reported in RA patients treated with Rituximab.

Immunosuppression also can lead to a theoretical risk of malignancy, as tumor surveillance by the immune system may be affected. Since RA patients have an increased risk of lymphoma secondary to the disease itself, the extent of the increased risk of developing a cancer such as lymphoma while taking immunosuppressive medications remains debatable.

A recent analysis of a German RA registry did not find an increased risk of malignancy, either hematologic or solid tumor, with the use of anti-TNF agents or anakinra,however, this included only four years of exposure data. Contrarily, an analysis of French patients on anti-TNF medications has shown an increased incidence of lymphoma in patients on adalimumab or infliximab.

The coexistence of other autoimmune diseases, such as Sjögren's syndrome, may also increase the likelihood of developing lymphoma, thus making it more difficult to determine the contribution from immunosuppressive medications.

Of all the non-joint-related complications of RA, cardiovascular disease is the most serious. It is the leading cause of death in both men and women in the U.S., and people with RA have twice the risk of most cardiovascular-related problems compared with the general population, including heart attack, strokes and atherosclerosis – hardening of the arteries caused by the build-up of plaque on the inner walls of the blood vessels. The risk of heart failure is also increased.

II. LITERATURE REVIEW

1. Preliminary evaluation of anti-inflammatory and anti-arthritic activity of S.lappa, A.speclosa and A.aspera.

Gokhale AB et. al., 2002 assessed anti-inflammatory and anti-arthritic activity of Saussurea lappa, Argyreia speciosa and Achyranthes aspera.The ethanolic extracts of the plants of the doses of 50, 100 and 200 mg/kg p.o. were screened for their effect on acute and chronic inflammation induced in mice and rats.S.lappa and A. speciosa were found to inhibit paw odema induced by carrageenan and Freund's complete adjuvant and to prevent accumulation of inflammatory cells in carrageenan –induced peritonitis at doses of 50-200 mg/kg.A. aspera

inhibited inflammatory responses at doses of 100-200 mg/kg.The studies reveal that the ethanolic extracts of S.lappa, A. speciosa and A. aspera posses anti-inflammatory and anti-arthritic activity and support the rationale behind the traditional use of these plants in inflammatory conditions.

2. Anti-inflammatory and anti-oxidant properties of Curcuma longa (turmeric) versus Zingiber officinale (ginger) rhizomes in rat adjuvant-induced arthritis

Ramadan G et.al, 2011 evaluated and compared anti-inflammatory and anti-oxidant properties of curcuma longa and zinger offficinale rhizomes in adjuvant induced arthritic rats.Both plants (at dose 200 mg/kg body weight) significantly suppressed the incidence and severity of arthritis by increasing/decreasing the production of anti-inflammatory/pro-inflammatory cytokines, respectively, and activating the anti-oxidant defense system.The anti-arthritic activity of turmeric exceeded that of ginger and indomethacin, especially when the treatment started from the day of arthritis induction.The percentage of disease recovery was 4.6-8.3% and 10.2% more in turmeric compared with ginger and indomethacin ($p<0.5$), respectively.

3. Evaluation of anti-nociceptive and anti-inflammatory effectof the crude extract of *I. pes-caprae* in mice

Souza et al., 2000 showed pronounce anti-nociceptive action of the hydro-alcoholic extract of *I. pescaprae* against two classical models of pain, neurogenic and inflammatory and support at least the traditional use of this plant for the treatment of dolorous processes.

4. Anti-Arthritic Potential of the Plant Justicia Gendarussa Burm F

Jaijesh Paval et.al, 2009 evaluated the anti-arthritic potential of the alcoholic extract of the plant Justicia gendarussa using the Freund's adjuvant-induced and collagen-induced arthritic rat models.The rats were treated with the ethanolic extract of Justicia gendarussa and with standard aspirin and showed significant arthritic activity similar to that of aspirin. The result obtained

suggests that the alcoholic extract of Justicia gendarussa exhibits significant anti-arthritic potential.

5. Anti-Arthritic Activity of Premna serratifolia Linn., Wood against Adjuvant Induced Arthritis

Rajendran R et.al, 2010 evaluated, anti-arthritic activity of ethanol extract of Premna serratifolia Linn. in Freund's adjuvant induced arthritis model.Loss in body weight during arthritis condition was corrected on treatment with ethanol extract and standard drug indomethacin.Biochemical parameters such as hemoglobin content, total WBC, RBC, erythrocyte and sedimentation rate were also estimated.The ethanol extract at the dose of 300 mg/kg body weight inhibited the rat paw edema by 68.32% which is comparable with standard drug indomethacin 74.87% inhibition of rat paw edema after 21 days.The ethanol extract of Premna serratifolia Linn wood possess a significant anti-arthriticactivity against adjuvantinducedarthritis andjustifying its therapeutic role in arthritic condition.

6. Evaluation of ant-arthritic activity potential of the methanolic extract of the aerial parts of costus speciosus

Shruti Srinivastava et al., 2012 assessed the anti-arthritic potential of *Costus speciosus* in Freund's adjuvant animals. The methanolic extract of CS in doses of 400 and 800 mg/kg showed 75.50% and 68.33% protection against increase in paw edema, respectively. CS showed dose-dependent action in all the experimental models.

7. Anti per oxidative effect of Withania somnifera root powder on liver lipid per oxidation and anti oxidant status in adjuvant induced arthritic rats

M.Rasool et.al.,2008 evaluated anti per oxidative effect of Withania somnifera root powder on liver lipid per oxidation and anti-oxidant status in adjuvant induced arthritic rats.Withania somnifera root powder (1000mg/kg) and indomethacin (3mg/kg) were orally administered for 8 days beginning 11 days after adjuvant injection.Results showed a significant decrease in the level of lipid peroxides, constituents with the increased enzymatic anti oxidants and depleted non enzymatic anti oxidant status in arthritic animals.

8. Evaluation of protective efficacy of Spirulina platensis against collagen-induced arthritis in rats.

Narendra Kumar et al., 2009 evaluated the protective efficacy of*Spirulina platensis* against collagen induced arthritis (CIA) in female wistar rats based on the changes in paw thickness, altered serum albumin, cholesterol, lipid per oxidation, alkaline phosphates and acid phosphatase activities and histology of paw joints.CIA rats were orally treated with 200 and 400 mg/kg per oral of *S. platensis* from 0 to 45 day. *S.Platensis* at 400 mg/kg per oral significantly elevates serum albumin and decreases the serum cholesterol, alkaline phosphatase and acid phosphatase activities, lipid per oxidation, paw thickness as well as normalize the joint histopathology of CIA rats.*S.platensis* at (400 mg/kg) significantly reduced the arthritic symptoms in treatment groups and this indicates that *S.platensis* has promising protective efficacy against CIA rats.

9. Anti rheumatoid activity of aqueous extract of piper longum on freund's adjuvant-induced arthritis in rats

Yende et al., 2010 studied anti arthritic activity of aqueous extract of the fruits of the plant piper longum in Freund's adjuvant Induced Arthritis Rats with the dose of 200 and 400 mg/kg p.o.The administration of extract reported significant reduction in paw swelling on 4^{th}, 8^{th}, 14^{th} and 21^{st} day after sub-plantar administration of Complete Freund's adjuvant.The paw swelling was measured as a volume displacement using digital plethysmometer.Furthermore, these results supported by radiographic analysis of affected knees of rats.From the result observed in the present investigation, it may be concluded that the aqueous extract of P.longum possesses potentially useful anti-arthritic activity in Complete Freund's Adjuvant model.

10. **Anti-arthritic activity of roots of Hemidesmus indicus R.Br.(Anantmul) in rats**

Alka metha et.al, 2012 evaluated the protective effects of hydro alcoholic and its fractions from roots of Hemidesmus indicus in complete Freund's adjuvant induced arthritis.Rats treated with hydro alcoholic extract (450 mg/kg p.o.) ethyl acetate (75 mg/kg p.o) chloroform (60 mg/kg.p.o) and residual fractions (270 mg/kg.p.o) showed significant decrease in physical and biochemical parameters compared with arthritic model rats.Hydro alcoholic extract and its ethyl acetate fraction of Hemidesmus indicus showed significantly higher anti-arthritic activity than

chloroform and residual fraction.Histopathological analysis demonstrated that both of hydro alcoholic extract and its ethyl acetate fraction and comparable anti-arthritic activity with methotrexate.The present study suggested that Hemidesmus indicus has protective activity against arthritis and the activity might be attributed to presence of terpenoid in hydro alcoholic extract, as well as in ethyl acetate fraction.

11. Protective effect of latex of *Calotropis procera* in Freund's Complete Adjuvant induced monoarthritis

Kumar VI et al, 2009 proved the protective effect of latex of *Calotropis procera* in Freund's Complete Adjuvant(FCA) induced monoarticular arthritis in rats. The effects of dried latex and its methanol extract following oral administration was evaluated on joint inflammation, hyperalgesia, locomotor function and histology at the time of peak inflammation. The results obtained suggested that the latex of *C.procera* has the potential to be used as an anti-arthritic agent.

12. **Anti-Rheumatic and Antioxidant activity of the extract of Stem bark of *Ficus bengalensis***

Manocha N et al., 2011 assessed the analgesic, anti rheumatic and anti oxidant activity of the methaolic extract of the bark of *Ficus bengalensis(MFB)* at doses of 100, 200 and 300 mg/kg(i.p) using the Freund's Complete Adjuvant induced arthritis model, the Formalin induced arthritis model and the Agar induced arthritis model . The extract produced marked inhibitory effect on edema especially on secondary immunological arthritis and caused graded inhibition of both phases of Formalin-induced pain. The present study validates the traditional use, demonstrating that the methanolic extract of the bark of this plant possesses dose-dependent anti-rheumatic activity in all the models with a possibility of acting through centrally and peripherally mediated activities.

III. SCOPE AND PLAN OF WORK

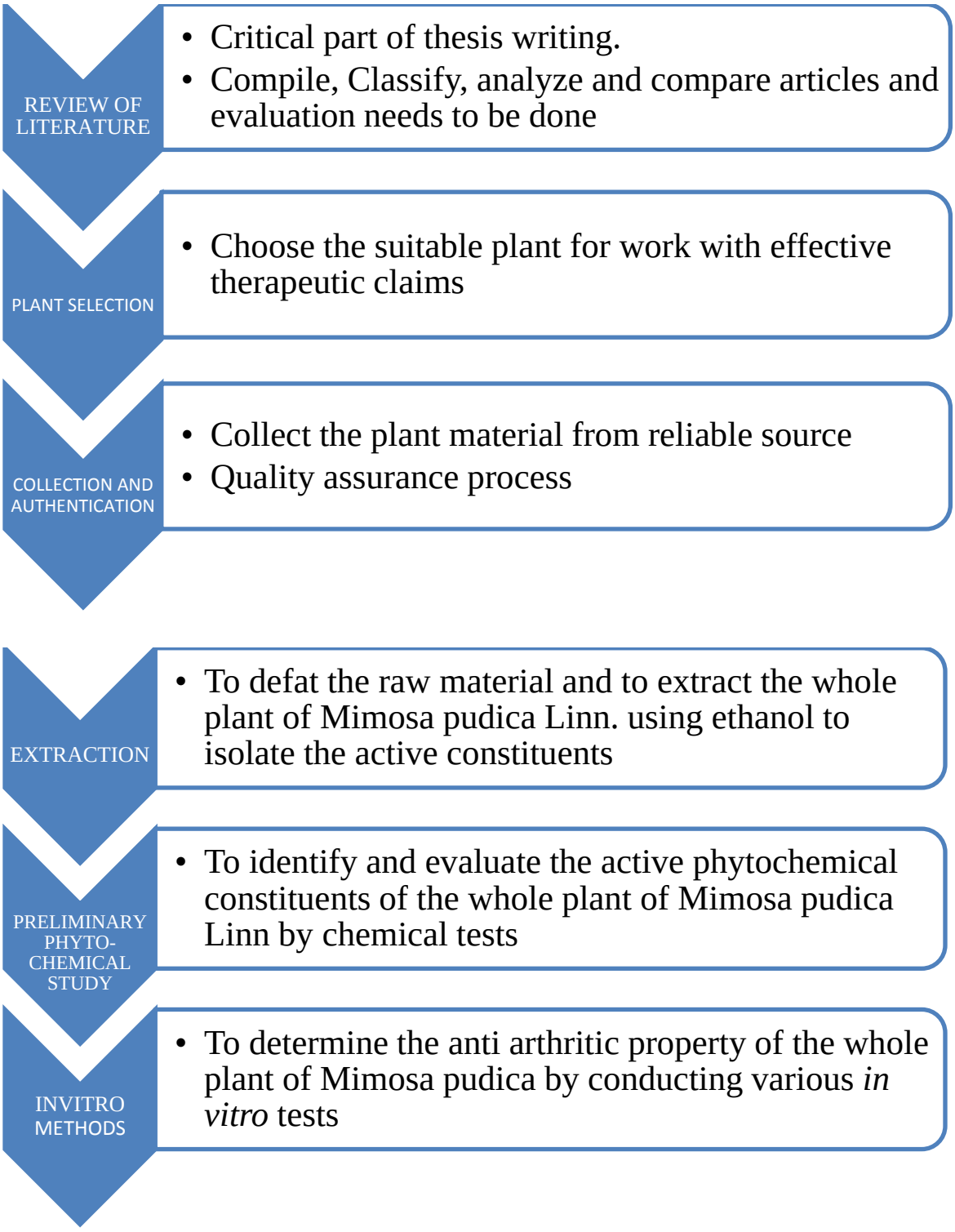
3.1 SCOPE OF WORK

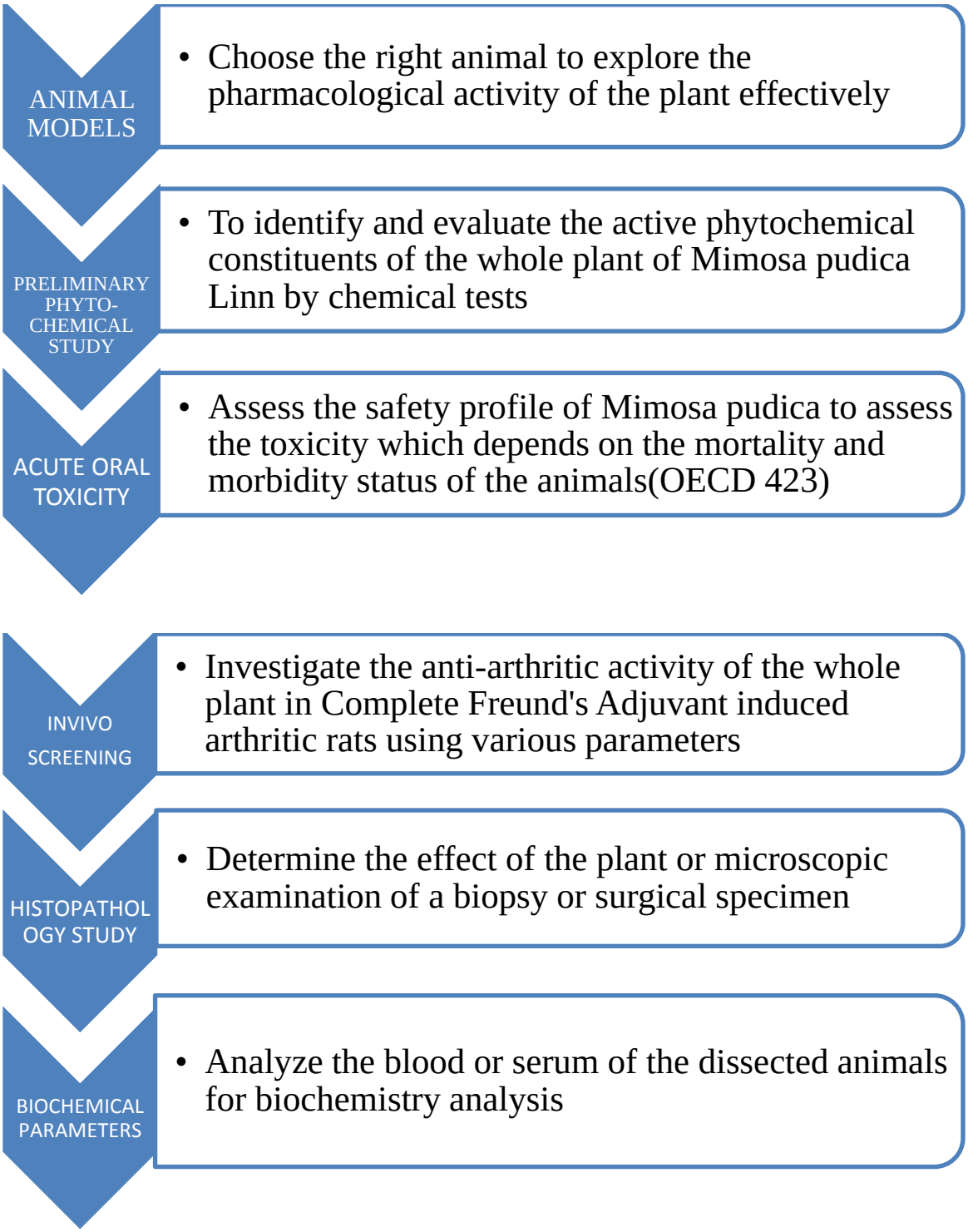
Rheumatoid arthritis is not a single disease where as it is an auto immune disease which affects the whole body system. Hence it is also known as a systemic disease where the joints are usually affected symmetrically. There are more than 100 types of arthritis and related conditions where the body's own immune system attacks the synovial membrane of the joint and causes synovitis. This results to various painful symptoms such as swelling and stiffness of joint and if left untreated the joint might permanently damage, resulting in deformity. Hence the goal of the treatment are as follows:

- Rectify the underlying cause of the disease i.e. to rectify or modify the deviated immune system of its abnormal reaction towards the healthy joint tissues to normal protective action.
- **Management of symptoms**: Homeopathy treatment targets the deviated immune system, which behaves in an abnormal way to healthy joint tissues and converts it to a healthy functioning immune system.
- **Technology**: Activated T-cells in the body are involved in the autoimmune response that leads to the joint inflammation and destruction often associated with RA. Abatacept (Bristol-Myers Squibb Pharmaceuticals Ltd) is a soluble fusion protein and is the first in a new class of agents called selective T-cell costimulation modulators. It acts by blocking one of the two signals needed for optimal T-cell activation, thereby interrupting the inflammatory process. Abatacept is administered via intravenous infusion.

  Abatacept is licensed in the US for the treatment of patients with moderately to severely active rheumatoid arthritis who have had an inadequate response to one or more DMARDs. It may be used as monotherapy or concurrently with other DMARDs except for TNF alpha inhibitors. Abatacept is not licensed in the UK, but an application for marketing authorisation has been submitted to the EMEA.

3.2 PLAN OF WORK


REVIEW OF LITERATURE
• Critical part of thesis writing.
• Compile, Classify, analyze and compare articles and evaluation needs to be done
PLANT SELECTION
• Choose the suitable plant for work with effective therapeutic claims
COLLECTION AND AUTHENTICATION
• Collect the plant material from reliable source
• Quality assurance process
EXTRACTION
• To defat the raw material and to extract the whole plant of Mimosa pudica Linn. using ethanol to isolate the active constituents
PRELIMINARY PHYTO-CHEMICAL STUDY
• To identify and evaluate the active phytochemical constituents of the whole plant of Mimosa pudica Linn by chemical tests
INVITRO METHODS
• To determine the anti arthritic property of the whole plant of Mimosa pudica by conducting various *in vitro* tests


ANIMAL MODELS
• Choose the right animal to explore the pharmacological activity of the plant effectively
PRELIMINARY PHYTO-CHEMICAL STUDY
• To identify and evaluate the active phytochemical constituents of the whole plant of Mimosa pudica Linn by chemical tests
ACUTE ORAL TOXICITY
• Assess the safety profile of Mimosa pudica to assess the toxicity which depends on the mortality and morbidity status of the animals(OECD 423)
INVIVO SCREENING
• Investigate the anti-arthritic activity of the whole plant in Complete Freund's Adjuvant induced arthritic rats using various parameters
HISTOPATHOLOGY STUDY
• Determine the effect of the plant or microscopic examination of a biopsy or surgical specimen
BIOCHEMICAL PARAMETERS
• Analyze the blood or serum of the dissected animals for biochemistry analysis

IV. MATERIALS AND METHODS

4.1 Collection of plant materials:

Figure G. Fresh herbs of Mimosa pudica Linn.

The plant *Mimosa pudicaLinn.*grows nearly throughout the tropical and sub-tropical parts of India. It is common in waste ground, particularly where the climate is moist and warm.

The plant is considered invasive in tropical climates.

- Temperature. Sensitive plant will grow indoors next to a sunny window with some direct sunlight.
- Soil. Sensitive plant should be planted in well-draining loamy soil enhanced with peat moss to improve drainage.

Figure H. Air-dried Mimosa pudica Linn.

Fresh and healthy plant materials were collected from Nellore district, Andhra Pradesh. It was identified and authenticated by Prof.P Jayaraman, Ph.D, Director, Institute of herbal botany, Plant anatomy and research centre, Chennai, Tamil Nadu, India. A copy of the authentication certificate is attached in**Annexure**.

Treatment

The collected plant materials were air dried at room temperature for about 5 days until it is free from moisture content. The dried materials were powdered by means of mechanical grinder and the resulting product was used for further study.

Plant profile

The term medicinal plants or herbs have been identified and played a significant role from the Stone Age period in maintaining good health and well-being of human. Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. A rich heritage of information and a large body of evidence was available in ancient scholastic works in the Indigenous systems of Medicine: *Ayurveda*, *Siddha*, *Unani* and *Homeopathy* drug.

For more than a decade, the pharmaceutical industry had disproportionate influence on the practice of medicine, as well as biomedical research. Meanwhile, the natural medicine has increasingly entered the spotlight, and a new study brings more attention to the latter. Herbal drugs or medicinal plants, their extracts and their isolated compound(s) have demonstrated

spectrum of biological activities and continued to be used as medicine in folklore or food supplement for various disorders.

An estimate suggests that about 13, 000 plant species worldwide are known to have use as drugs. The trend of using natural products has increased and the active plant extracts are frequently used for new drug discoveries and for the presence of active phyto-therapeutic materials.

MIMOSA PUDICALINN.

Figure I. Mimosa pudica Linn.

"Mimic" means to allude and "pudica" means bashful, results the name *Mimosa pudica* to that plant.[1] In legume family, *Mimosa* is one of the largest genera which distribute more than 500 species. *Mimosa pudica L.* (Mimosaceae) is a common plant in moist waste ground, lawns, open plantations and weedy thickets. It is native from Middle America and now widely distributed in all tropical areas[53]. Mimosa Pudica seeks attention of the researchers worldwide for its pharmacological activities such as anti diabetic, antitoxin, anti hepatotoxin, antioxidant and wound healing activity. *Mimosa pudica Linn* locally known as chuimui (Eng: Touch me not) in Malwa region, India, is traditionally used as an agent for birth control among rural people. This creeping perennial herb has been mentioned as a tribal medicine all over India[54].

Traditionally M. pudica is used in the treatment of headache, migraine, insomnia, diarrhea, dysentery, fever, piles and fistula. Roots in the form of decoction are used to treat urinary complaints and in diseases arising from corrupt blood and bile. The paste of the leaves is applied to glandular swelling and dressing for sinus. Only few pharmacological studies have been reported on leaves of M.pudica like hypoglycemic [55] and anticonvulsant activity. Leaves of this plant has been used to control swelling and dressing of wounds. The claim that the anti-inflammatory activity of M. pudica leaves is speculative and has not yet been documented. In the present study an attempt has been made to evaluate the anti-inflammatory efficacy of M. pudica leaves in validated models of rates.

Botanical Information

Family: Leguminosae

A diffuse under shrub, 50-90 cm high, native of tropical America and naturalized nearly throughout topical and sub-tropical parts of India. Stem and rachis clothed with prickles; leaves bipinnate: pinnae 2-4, digitatively arranged, with 10 pairs of leaflets; flowers in pinkish globose heads; pods small, flat, straw coloured with many bristles; seeds 3-5.

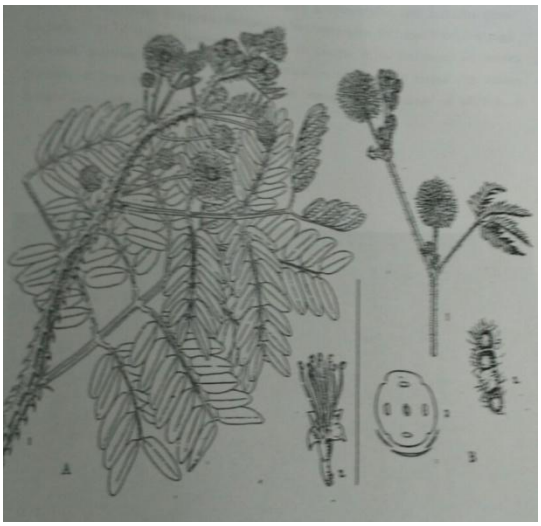


Figure J. Parts of M. pudica

This is the second biggest family among the dicotyledons (being second only to Compositae), and has varying characteristics. As such, it has been divided into the following sub-families; Papilionaceae, Caesalpinieae and Mimoseae. The division is primarily based on the

characteristics of the corolla and stamens. All these sub-families are well represented in India.From an economic standpoint, this is one of the most important families. It probably ranks second to Gramineae in the order of importance.

The picture below depicts the following parts:

A_1- Stem with aerial parts

A2- Androecium

B1- Flowering top

B2- Fruit

B3- Floral diagram

Synonyms:

Language	Vernacular name
Sanskrit	Namaskari
Ayurveda	Lajjalu
Hindi	Chui mui
	Lajawanti
Tamil	Tottal sinungi
Bengali	Lojjaboti
Malayalam	Thottavadi
Marathi	Lazalu
Kannada	Muttidare muni
Indonesia	Putri malu

Myanmar (Burma)	Hti ka yoan
Latin	Pudica
Malaysia	Pakok semalu
European	Naa - me – toque
	Sensitive
	Dormideira
Spanish	Mori – vivi

Active constituents[56]

- Crocetin dimethyl ester and tannin have been isolated from the plant.
- The mucilage from seed is composed of D-xylose and D-glucoronic acid 4-*O*-(3, 5-dihydroxybenzoic acid)-b-D-glucoronide. It has four flavones namely 7, 8, 3', 4'-tetrahydroxyl-6-C-[alpha-l-rhamnopyranosyl-(1→2)]-b-D-glucopyranosyl flavone (I); 5, 7, 4'-trihydroxyl-8-C-[a-l-rhamnopyranosyl-(1→2)]- D-glucopyranosyl flavone (III) and catcher (IV).
- A saponin and a bufadienolide were reported in *M. pudica* seeds.
- P-Flavanoids, Phenolic constituents, Saponins, Glycosides, Gums, Tubuline, Phytosterol, Adrenaline like substances - leaf extract.
- Green yellow fatty oil -17%, Terpenoids, Coumarins, Quinines , derivatives of 4-α-(b-d-glucopyranosyl-6-sulphate) gallic acid , c- glycosylflavone, Phenolic ketone, Jasmonic acid, Nor-epinephrine, d-pinitol (3-mono-methyl ether of inositol), b-sitosterol.
- Coumaric acid is a common plant constituent. Coumaric acid derivatives act as leaf-opening substances in other nyctinastic plants. C-glycosyl flavones present in aerial part.
- The leaves contain beta sitosterol and phenolic ketones. Oil extract contains amino acid and aminoacid derivatives like N-dl-Alanylglycine, dl-Alanyl-dl- Valine, d-Alanin, dl-Alanin ethyl ester, dl-Alanyl-dl-Valine and 1-Alanine ethyl amide .oil extact possess

derivatives of fattyacid like 9, 12-Octadecadienoic acid (Z, Z), methyl ester, 11, 13-Eicosadienoic acid.

- The other constituents present in the oil extract are methyl ester, 2-methylamino-N-phenyl-acetamide, 1-Octanamine, N-methyl, 1-Butanamine, N-methyl, Meglumine, 2-methylamino-n-phenyl acetamide, - 1, 3-Dioxolane-4-methanol, 2, 5-Dimethoxy-4-(methylsulphonyl) amphetamines, 9.12-Octadecadien-1-ol and 11, 13-Eicosadienoic acid, methyl ester.[35]
- The Structures of selected phytochemicals (mimosine, tyrosine 3,4-dihydroxypiridine, mimosinamine, mimosinic acid) from Mimosa pudica.

Use of chemical constituents:

It is reported to contain alkaloid, glycoside, flavonoid and tannins. Its extract immobilizes the filariform larvae of Strongyloides stercoralis in less than one hour. In contemporary medicine, Mimosa pudica is being investigated for its potential to yield novel chemotherapeutic compounds. It contains an alkaloid called mimosine, which has been found to have potent antiproliferative and apoptotic effects. Aqueous extracts of the roots of the plant have shown significant neutralizing effects on the lethality of the venom of the monocled cobra (Naja Kaouthia). It appears to inhibit the myotoxicity and enzyme activity of cobra venom. M. pudica contains mimosine which is a toxic alkaloid. Adrenalin like substance has been identified in the extract of its leaves. Some workers have reported the presence of Crocetin dimethyl Easter in the extract of the plant. Roots contain tannin up to 10 per cent. Seeds contain a mucilage which is composed of d-xylose and d-glucuronic acid. The plant extract contains green yellow fatty oil up to 17 per cent. The plant is reported to contain tubuline and a new class phytohormone turgorines is found to be active in the plant. The periodic leaf movement factors are reportedly the derivatives of 4-o-(b-D-glucopyranosyl-6-sulphate) gallic acid.

Leaflet movement physiology[57]

Figure K. Drooping leaves of M. Pudica

The leaflets fold together in the early evening and reopen at sunrise. It is called bashful or sensitive because the leaflets fold together on touching, warming and shaking. The phenomenon is called seismonastic movement due to a rapid change in turgor pressure and changes in membrane permeability in the pulvini cells in the leaf regions with rapid movement of calcium ions. At night, the leaves also fold and bend, termed nyctonastic movements (reaction to absence of light). Seismonastic Movement / Actin Cystoskeleton: Study showed fragmentation of actin filaments occurring during bending was invovled in the regulation of movement. The effect of phosphatase inhibitors on the actin cytoskeleton affects dynamic reorganization of actin filaments and causes the seismonastic movement.Mimosa pudica is a thigmonastic plant that reacts in response to stressors such as electrostimulation, wound, wind, vibration, touch, drought, change of illumination, and hot or cold stimuli. Mimosa pudica reacts to stimulation by closure of leaves and descent of petiole. The anatomy of M. pudica is unique and contributes to the bioelectrochemical response mechanism of the plant. The propagation of action potentials is a signaling mechanism in M. pudica. The action potentials that occur in plants have many of the same properties as action potentials that occur in animals including the allor-nothing law, threshold potential, and refractory period. Tactile stimulation of M. pudica induces transmission of an action potential that stops at the base of a single pinna with no further transmission occurring, leaving leaflets from neighboring pinnae unfolded.

Applications of M. pudica in Traditional Healthcare System[58]

Ayurveda has declared that its root is bitter, acrid, cooling, vulnerary, alexipharmic, and used in the treatment of leprosy, dysentery, vaginal and uterine complaints, inflammations, burning sensation, asthma, leucoderma, and fatigue and blood diseases. Unani Healthcare System its root is resolvent, alternative, and useful in the treatment of diseases arising from blood impurities and bile, bilious fevers, piles, jaundice, and leprosy etc. Decoction of root is used with water to gargle to reduce toothache. It is very useful in diarrhea (athisaara), amoebic dysentery (raktaatisaara), bleeding piles and urinary infections. It arrests bleeding and fastens the wound healing process. It is mainly used in herbal preparations for gynecological disorders. It has been said to have medicinal properties to cure skin diseases. It is also used in conditions like bronchitis, general weakness and impotence. It is also used to treat neurological problems. The content of M. pudica has a capacity of arresting bleeding and it fastens the process of healing of wounds. It is recommended in diarrhea, amoebic dysentery and bleeding piles. It is also used in herbal preparations of gynecological disorders. Its extract can cure skin diseases. Some herbal doctors recommend it for bronchitis, general weakness and impotence. All the five parts of the plant (that is the PANCHANG) - leaves, flowers, stems, roots, and fruits are used as medicines in the traditional healthcare systems. In India, different parts of the plant have been in popular use for treating various ailments since long.

Recent researches show that the extract of this plant can be used for checking child birth. Some authors have reported that this herb can replace contraceptive pills if researches are done properly. According to different researches done so far, Mimosa Tenuiflora bark is used to relax the mind, and relieve depression, mental distress, irritability, severe palpitations, and amnesia. It is a mood enhancer and improves circulation of the blood. Some believe Mimosa can reduce the onset of baldness. Due to its ability to promote healthy cell growth, Tepezcohuite is used in shampoos, creams, capsules, and soaps. In Ayurvedic and Unani medicine, Mimosa pudica root is used to treat bilious fevers, piles, jaundice, leprosy, dysentery, vaginal and uterine complaints, inflammations, burning sensation, fatigue, asthma, leucoderma, and blood diseases. In Western medicine, Mimosa root is used for treating insomnia, irritability, premenstrual syndrome (PMS), menorrhagia, hemorrhoids, skin wounds, and diarrhea. It is also used to treat whooping cough and fevers in children, and there is some evidence to suggest that ***Mimosa is effective in relieving the symptoms of rheumatoid arthritis***. Its consumption is not recommended to pregnant or nursing ladies. Due to these reports, it seems to be best to consult a physician before

using Mimosa internally. Researches regarding safety in young children or those with severe liver or kidney disease have not been found.

4.2 GLASSWARE AND CHEMICALS

The glassware and the laboratory instruments used for study were of analytical grade procured from approved organization.

Chemicals	Company Name
A-Naphthol	Himedia, Mumbai

Ruthenium red Phenolphthalein	
Sulphuric acid Sodium hydroxide Nitric acid Ammonia Hydrochloric acid Glacial acetic acid	Merck, Mumbai
Fehling's solution A & B Benedict's reagent Dragendroff's reagent Wagner's reagent Mayer's reagent Hager's reagent	Sigma chemicals, USA
Copper sulphate Lead acetate Potassium dichromate Magnesium turnings Tin metal	Qualigens Fine chemicals, Mumbai
Ferric chloride Acetic anhydride Pyridine Glacial acetic acid Ninhydrin	Loba Chemicals, Mumbai
Toluene Sodium nitroprusside Thionyl chloride Ethyl acetate Dimethyl amine Acetone	Fisher inorganic and Aromatic Ltd, Chennai
Ethanol	S D Fine Chemical Laboratory, Mumbai

Methanol N-Propanol Formic acid Cyclohexane Acetonitrile Chloroform	

INSTRUMENTS USED

Instruments	Company name
Analytical balance	Sartorius Ltd. Germany
Digital ph meter	Systronics
Ultra sonicator	Lark Park. Ltd, New Delhi
Centrifuge 412 LAG	Remi instrument Ltd, Mumbai
IKAT 25 Teflon homogenizer	Ultra Turrax Ltd, Germany
Magnetic stirrer	Remi instrument Ltd, Mumbai
Orbit shaker and incubator	Labnet scientific services, Chennai
Deep Freezer	Whirlpool
Rotavapor	Buchi, RE121
Hot air oven	Bionics, Delhi
Thermostatic water bath	Bio Technics, Mumbai

4.3 STUDY ON ORGANOLEPTIC CHARACTERISTICS

Ash value

The residue remaining after incineration is the ash content of the drug which consists of inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it as a form of adulteration. Ash value is a basis to judge the identity or purity of crude drugs.

The percentage of ash in medicinal plant materials is determined by three different methods:

1) Determination of total ash
2) Determination of acid Insoluble ash
3) Determination of sulphated ash

Total ash

There are two types of ash which remains after igniting the plant material and they are physiological ash and non physiological ash. The erstwhile is derived from the plant tissue while the hindmost is obtained by igniting the extraneous matter like sand and soil adhering to the surface of the plant material. These two values of ash constitutes the total ash value.

Determination of total ash

About 3 gm of the powdered drug was accurately weighed in a silica crucible which was previously dried and weighed. The powdered material was spread to a thin layer in the crucible and then ignited gradually by increasing the temperature not exceeding 450^0C until it is free from carbon. Cooled in a desiccator and weighed.

The procedure was repeated to the constant weight and the percentage of the total ash was calculated with reference to the air dried drug. The total ash value of whole plant of Mimosa pudica (Linn) is recorded in Table No 1.

$$\text{Total ash value of the sample} = \frac{Z-X}{Y} X\ 100$$

Where,

Weight of the empty crucible= Z,

Weight of the crucible with ash= X,

Weight of the plant material= Y

Determination of acid insoluble ash

The ash obtained from the determination of total ash was boiled with 25ml of dilute hydrochloric acid for five minutes. The insoluble matter was collected on an ash-less filter paper

and washed with hot water. The insoluble ash was transferred into a pre-weighed silica crucible, was ignited, cooled and kept in desiccators. The procedure was repeated to get constant weight.

The percentage of acid insoluble ash was calculated with reference to the air dried drug.

$$\text{Acid insoluble ash value of the sample} = \frac{100}{Y}\ X\ A$$

Where,

Weight of the residue obtained = A,

Weight of the plant material= Y

Determination of sulphated ash

About 1gm of the powdered drug was weighed accurately in a silica crucible. The crucible was heated to redness for 10 minutes, gently at first until the drug was thoroughly charred. Allowed to cool in a desiccator and weighed. The crucible was cooled and the residue was moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800^0c $\pm 25^0$c until all black particles had disappeared. The ignition was conducted in a place protected from air currents. The crucible was allowed to cool, few drops of sulphuric acid was added and again heated. The ignition was carried out as before, allowed to cool and weighed. The operation was repeated until two successive weighing did not differ by more than 0.5mg.The percentage of sulphated ash was calculated with reference to the air dried.

Extractive value

The extracts obtained by exhausting crude drugs are indicative of approximate measures of their chemical constituents and the percentage of extractable constituents present in a given plant material is known as extractive value of the particular plant material. Taking into the consideration, the diversity in chemical nature and properties of contents of drugs, various solvents such as water and alcohol are used for determination of extractives. Extractive value of the sample was determined as the method of WHO.

$$Extractive\ value(\%) = \frac{Weight\ of\ the\ residue\ obtained}{Weight\ of\ the\ plant\ material\ taken} \text{x } 100$$

Water soluble extractive

This method is applied to assess the content and percentage of water soluble constituents of crude drugs such as tannins, sugars, mucilage and plant acids.

Alcohol soluble extractive

This method can be used to determine the presence and amount of alcohol soluble constituents such as resins, tannins and alkaloids.

i. Determination of water soluble extractive

Macerated about 5 gm of the air dried coarse powder of whole plant of Mimosa pudica (Linn) with 100 ml of chloroform water in a glass stoppered volumetric flask for 24 hours, shaken frequently for the first 6 hours and allowed to stand for 18 hours. Filtered rapidly, taking precautions against loss of the solvent. 25mlof the filtrate was transferred to a tared flat bottomed dish and evaporated to dryness. Dried at 105^0 C for 6 hours, cooled in a desiccator for 30minutes and weighed immediately without delay. The percentage of water soluble extractive value was calculated with reference to the air dried drug.

The water soluble extractive value of the whole plant of Mimosa pudica (Linn) is recorded in Table No 2.

ii. Determination of ethanol soluble extractive

5gm of the powdered air dried coarse powder of whole plant of Mimosa pudica (Linn) was weighed accurately and taken in a glass stoppered volumetric flask. 100ml of 95% ethanol was added to the flask and the plant material was macerated for 24 hours, shaking intermittently for the first 6 hours. It was then allowed to stand for 18 hours. Thereafter, it was filtered rapidly without loss of solvent. Evaporated 25ml of the filtrate to dryness in a tared flat bottom shallow dish, dried at 105^0 C for 6 hours. Cooled in a desiccator for 30 minutes and immediately weighed. The recorded value of the ethanol soluble extractive value of the whole plant of Mimosa pudica (Linn) is mentioned in Table No.2

4.4 PREMILINARY PHYTOCHEMICAL EVALUATION

Introduction

The importance of medicinal plant in drug development is known to us and humans have used them for different diseases from the beginning of human history.

Traditional folk treatment from wild plants has always guided researchers to search for novel medications to develop healthy life for humans and animals.

In addition, some medicinal plants are still obscured within the plant which need to be scientifically evaluated.

Preliminary screening of phytochemicals is a valuable step, in the detection of the bioactive principles present in medicinal plants and subsequently may lead to drug discovery and development. In the present study, chief phytoconstituents of the medicinal plant was identified in order to relate their presence with bioactivity of the plant.

Phytochemical analysis is divided into two categories:

1. Qualitative
2. Quantitative

Standard procedures:

1. Sofowara(1993)
2. Trease and Evans(1989)
3. Harborne(1973)

4.5 PREPARATION AND METHOD OF EXTRACTS

About 750 grams of powdered whole plant of Mimosa pudica (Linn.) was taken in a 5000ml of round bottom flask and extracted using cold extraction method for 12 days using ethanol (50%) with occasional stirring. The ethanol extract was filtered through Whatman filter paper to remove the impurities present. The ethanolic extract was concentrated by vacuum distillation, cooled and placed in desiccator to remove the excessive moisture.

Quantity of extract obtained- **154.21 grams** from **750 grams** of the raw material.

Qualitative Phytochemical Screening:

The extracts of Mimosa pudica (Linn.) was subjected to the preliminary phytochemical analysis using following chemical tests for the identification of various active constituents present.

Following are the tests carried out to identify the possible phytoconstituents and the results are tabulated below:

A. Detection of carbohydrates

1. **Molisch's Test:** To 2 ml of the extract, add 1 ml of α-naplathol solution and concentrated sulphuric acid through the sides of test tube. Purple or reddish violet colour junction between the two liquids reveals the presence of carbohydrates.

2. **Fehling's Test:** To 1ml of the extract, add equal quantities of Fehling's solution A and B. Upon heating, a brick red precipitate indicates the presence of carbohydrates.

3. **Benedict's Test:** To 5 ml of Benedict's reagent, add 1 ml of extract solution and boil for 2 minutes and cool.Formation of a red precipitate shows the presence of carbohydrates.

B. Detection of alkaloids

1. **Dragendroff's Test:** To 1 ml of the extract, add 1 ml Dragendroff's reagent. An orange red precipitate shows the presence of alkaloids.

2. **Wagner's Test:** To 1 ml of the extract, add 2 ml of Wagner's reagent. Formation of a reddish brown precipitate specifies the presence of alkaloids.

3. **Mayer's Test:** To 1 ml of the extract, add 2 ml of Mayer's reagent. A dull white or creamy precipitate declares the presence of alkaloids.

4. **Hager's Test:** To 1 ml of the extract, add 3 ml of Hager's reagent. Yellow precipitate confirms the presence of alkaloids.

C. Detection of proteins and free amino acids

1. **Biuret Test:** To 1 ml of the extract, add 1 ml of 40% sodium hydroxide solution and 2 drops of 1% copper sulphate solution. Formation of violet colour indicates the presence of proteins.
2. **Xanthoprotein Test:** To 1 ml of the extract add 1 ml of concentrated nitric acid. A white precipitate is formed, it is boiled and cooled.Then 20% of sodium hydroxide or ammonia is added. Orange colour indicates the presence of aromatic amino acids.
3. **Lead Acetate Test**: To 1 ml extract, 1 ml of lead acetate solution is added. Formation of dull white precipitate indicates the presence of proteins.

4. **Ninhydrin Test:** Add two drops of freshly prepared 0.2% ninhydrin reagent to the extract solution and heat. Development of blue colour reveals the presence of protein peptides or amino acids.

D. Detection of Tannins and Phenolics

1. To 1 ml of the extract, add ferric chloride solution. Formation of a dark blue or greenish black colour product shows the presence of tannins.

2. To the extract, add potassium dichromate solution. Formation of a precipitate shows the presence of tannins and phenolics.

E. Detection of Flavonoids

1. **Shinoda's Test :** To 1 ml of the extract, add magnesium turnings and 1-2 drops of concentrated hydrochloric acid. Formationof red colour shows the presence of flavonoids.

F. Detection of Triterpenoids

1. Dissolve two or three granules of tin metal in 2 ml thionyl chloride solution.Then add 1 ml of the extract into the test tube. The formation of a pink colour indicates the presence of triterpenoids.

G. Detection of steroids

1. **Libermann Burchard Test:** Dissolve the extract in 2 ml of chloroform in a dry test tube. Add 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The solution becomes red, then blue and finally bluish green indicates the presence of steroids.

2. **Salkowski Test:** Dissolve the extract in chloroform and add equal volumes of concentrated sulphuric acid. Formation of bluish red to cherry red colour in chloroform layer and green fluorescence in the acid layer represents the steroid components in the tested extract.

3. **Liebermann's reaction :**Mix 3 ml of extract with 3 ml acetic anhydride, heat and cool, add few drops of concentrated sulphuric acid, blue colour appears.

H. Detection of Saponins

1. About 1 ml of extract is diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. A 1 cm layer of foam indicates the presence of Saponins.

I. Detection of Fixed Oils

1. **Spot Test:** Press a small quantity of extract between two filter papers. Oil stains on paper indicates the presence of Fixed Oils.

2. **Saponification Test:** To 1 ml of the extract, add few drops of 0.5 N alcoholic potassium hydroxide along with a drop of phenolphthalein. Heat the mixture on a water bath for 1 -2 hours. The formation of soap or partial neutralization indicates the presence of Fixed Oils.

J. Detection of glycosides

1. **Legal Test:** Dissolve the extract in pyridine and add sodium nitroprusside solution to make it alkaline. The formation of pink red to red colour shows the presence of glycosides.

2. **Baljet Test:** To 1 ml of the test extract, add 1 ml sodium picrate solution and transformation of yellow to orange colour reveals the presence of glycosides.

3. **Borntrager's Test:** To 1 ml of extract solution, add a few ml of dilute sulphuric acid. Boil, filter and extract the filtrate with chloroform. The chloroform layer is treated with 1 ml of ammonia. The formation of red colour shows the presence of Anthraquinone Glycosides.

4. **Keller Kiliani Test:** Dissolve the extract in acetic acid containing traces of ferric chloride and transfer to a test tube containing sulphuric acid. At the junction, formation of a reddish brown colour, which gradually becomes blue, confirms the presence of glycosides.

K. Detection of gums

1. Hydrolyse the test solution using dilute hydrochloric acid. Perform Fehling's or Benedict's test. Red colour is developed.

L. Detection of mucilage

1. Powdered drug swells in water.
2. Powdered drug material shows red colour with ruthenium red.

4.6 ANALYTICAL METHODS

Fluorescence analysis

Fluorescence is the emission of light by the organic substance that has absorbed light or other electromagnetic radiation. It is a form of luminescence which occurs frequently in nature in some minerals and in various biological states. Fluorescent characteristics of the raw plant powders and after treating them with chemical reagents were observed in day light (visible) as well as under UV radiation.

Chromatographic study

Thin Layer Chromatography (TLC): The TLC identity tests provided in the chromatographs include identification of the drug based on its major chemical constituents.

TLC of Alkaloids

Preparation of Extracts

Preparation of mother extract

1 gm of 50% ethanolic extract of whole plant of Mimosa pudica (Linn) was dissolved in 50 ml of 50% ethanol by shaking for 15 minutes at 60^{o} C with 50 ml of 50% ethanol. It was

filtered through Whatman filter paper, and excess of ethanol was added into the marc. The resultant filtrate was made upto 100 ml with 50% ethanol to obtain a concentration of 10mg/ml.

Preparation of alkaloidal fraction

1 gm of 50% ethanolic extract of Mimosa pudica (Linn) (whole plant) was moistened with 1ml of 10% ammonia solution and then extracted by shaking for 15 minutes at 60^{o} C with 5ml of methanol. It was filtered through Whatman filter paper, and excess of methanol was added into the marc. The resultant filtrate was made upto 100 ml with methanol to obtain a concentration of 10mg/ml.

Preparation of Methanolic fraction

1 gm of 50% ethanolic extract of whole plant of Mimosa pudica (Linn) was dissolved in 50 ml of methanol by shaking for 15 minutes at 60^{o} C with 50 ml of methanol. It was filtered through Whatman filter paper, and excess of ethanol was added into the marc. The resultant filtrate was made upto 100 ml with methanol to obtain a concentration of 10mg/ml.

Preparation of ethyl acetate fraction

1 gm of 50% ethanolic extract of whole plant of Mimosa pudica (Linn) was dissolved in 50 ml of ethyl acetate by shaking for 15 minutes at 60^{o} C with 50 ml of ethyl acetate. It was filtered through Whatman filter paper, and excess of ethyl acetate was added into the marc. The resultant filtrate was made upto 100 ml with ethyl acetate to obtain a concentration of 10 mg/ml.

Stationary phase – Silica gel GF plate

Mobile phase

Chromatography was tried with the following solvent system.

a. Toluene-ethyl acetate-dimethyl amine (70:20:10)
b. Chloroform-dimethyl amine(90:20)
c. Toluene-acetone-ethanol-Concentrated ammonia (40:40:6:2)
d. Acetone-water- Concentrated ammonia (90:7:3)

e. Chloroform-methanol (85:15)
f. Toluene-methanol (86:14)
g. N-Propanol-formic acid-water(90:1:9)
h. Cyclohexane-chloroform-glacial acetic acid(45:45:10)
i. Acetonitrile-methanol (4:6)

The solvent system Acetonitrile-methanol gave more satisfactory spot in the ratio 4:6 for alkaloid.

Detection of component

Visualising agent: Dragendorff reagent

Colour of the spot: reddish brown

Application of the extract

All the extract 10 mg/ml was taken in a capillary tube and spotted on the plate keeping a distance of about 2 cm above the base of the plate.

Development of the chromatogram

The plates were then developed in TLC chamber previously provided and saturated with appropriate solvent system. After the development of the chromatogram, the plates were removed treated with visualizing agents and examined for the presence of the different spots. The R_f value were calculated and tabulated in Table No 5.

V. STUDY PLAN

5.1 Procurement of animals

Adult albino female rats of Wistar strain (150-200 g) were collected from C.L. Baid Metha college of Pharmacy were used in the pharmacological and toxicological studies. The animals were maintained and acclimatized to animal house conditions using paddy husk bedding at 25 ± 2^0C temperature and 50 ± 5% humidity with day night cycle (12 ± 1 h) in solid bottomed polypropylene cages. The rats were fed with balanced rodent pellet diet from Poultry Research Station, Nandanam, Chennai, India and water ad libitum was provided throughout the experimental period. The animals were sheltered for a week and prior to the experiment they were acclimatized to laboratory temperature. Food was withdrawn 2 hours before and during experimental duration. The protocol was approved by Animal Ethics Committee constituted for the purpose as per CPCSEA Guideline.

TOXICITY STUDIES

5.2 Acute oral toxicity study

Acute toxicity studies were conducted in female albino rats (150-200 g) body weight by *Staircase Method* of Ghosh [58]. The Experimental animals were subjected to acute oral toxicity studies as per revised OECD Organization of Economic Co-operation and Development guidelines (OECD No. 423) and acute class method.

Principle:

The acute oral toxicity class method is a stepwise procedure with 3 animals of single sex to assess the short term toxicity of test substance. An average of 2-4 steps may be involved to decide the acute toxicity of the plant material which depends on the mortality and morbidity status of the animals. The procedure is reproducible where usage ofanimals are minimal (Test Guidelines 420 and 425).

Procedure:

The animals were segregated into 5 groups containing 6 animals each. The method is established on bio metric evaluation with fixed doses (5, 100, 500, 2000 mg/kg P.O) to enable a substance to be ranked for classification purposes according to Globally Harmonized System (GHS) which causes acute toxicity.

The animals were fasted over-night prior to dosing (provided only water). Following the period of fasting, the animals should be weighed and the ethanolic extracts of Mimosa pudica (Linn.) was administered to the groups orally by gastric intubation canula. Single oral dose of the plant extract were devoid of any toxicity up to 2000 mg/kg body weight in albino rats for 14 days. The animals were observed at an hourly basis for 24 hours to assess the behavioral changes, locomotion, convulsions and mortality.

If mortality was observed in 5 or 6 animals among 6 animals, then the dose administered was assigned as a toxic dose. If mortality was observed in 3 animals, then the same dose was repeated again to confirm the toxic dose.

The optimum conditions for experiments were decided on the basis of pilot experiments carried out using five animals per group. 200 mg/kg (P.O) and 400 mg/kg (P.O) was taken as the therapeutic oral dose (low and high dose) for the compound.

Animals were weighed before and after the acute toxicity experimental studies. The following clinical observations of the animals were observed and recorded:

- Toxic signs

- Changes in body weight
- *Cage-side* observations
    - Feces colour
    - Skin and fur colour
    - Eyes and mucous membrane
    - Biting
    - Arousal
- Physical examination
    - Cardiovascular system
        - ✓ Heart rate
    - Autonomic nervous system
        - ✓ Lacrimation
        - ✓ Salivation
        - ✓ Fecal boluses
        - ✓ Urinary incontinence
    - Central nervous system
        - ✓ Tremors
        - ✓ Convulsions
        - ✓ Vocalization
        - ✓ Motor activity level
        - ✓ Rearing
        - ✓ Gait description
        - ✓ Response
            - ➢ Click response
            - ➢ Approach response
            - ➢ Touch response
            - ➢ Tail pinch response
        - ✓ Reflux
            - ➢ Flexor reflux
            - ➢ Extensor reflex
            - ➢ Pinna reflex
            - ➢ Pupillary reflex
        - ✓ Body posture

- Fore limb hoping
- Hind limb extension
- Surface righting reaction
- Aerial righting reaction
- Proprioceptive reaction

5.3 *INVIVO* ANTI-ARTHRITIC STUDY

Chemicals

Freund's complete adjuvant (Sigma Aldrich) and all other chemicals and reagents used for the study were of analytical grade procured from approved organization.

Animals

Female Wistar rats of body weight 150–200 g were used for the study. The animals were maintained under standard environmental conditions and were fed with standard pellet diet and water ad libitum. All the experimental procedures were carried out in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) guidelines and all the experimental procedures were approved by IAEC/L/02/CLBMCP/2017 (Approval no.).

Induction of arthritis

To induce arthritis, animals were first anesthetized with a small amount of ether vapor, then a single injection of 0.2 ml Complete Freund's adjuvant dissolved in mineral oil (sterile) was injected delicately into the sub-plantar region of hind paw.

Treatment regimen

The anti–arthritic activity was performed according to Jubie et al. [59] method. After classifying and grouping animals according to their weight, each animal was marked and placed in a cage with a letter identifying the cage.

The animals were divided into five groups of six animals each. Each group was given a dose schedule as follows:

1. Group I -Vehicle control, 1% w/v DMSO, p.o; (nonarthritic);
2. Group II - Negative control (0.1ml Complete Freund's adjuvant);
3. Group III -Arthritic animals treated with standard, 0.75 mg/kg Methotrexate, p.o;
4. Group IV - Arthritic animals treated with 200 mg/kg of Mimosa pudica(Linn.), p.o;
5. Group V -Arthritic animals treated with 400 mg/kg of Mimosa pudica(Linn.), p.o;

Vehicle control animals were given 1% w/v of DMSO solution daily.

Negative control group was given a single injection of 0.2 ml Complete Freund's adjuvant in mineral oil into the sub-plantar region of hind paw on day 1 under light ether anesthesia.

The stock solution was prepared on a daily basis for the treatment of low dose, high dose and standard dose animals depending upon the body weight of animals. The plant extract was diluted in DMSO because of its solubilizing effect and no solubility in water. Freshly prepared drug was introduced into the group of animals through oral administration using oral cannula.

The drug treatment was continued with the respective groups for 40 days.

Every day animals were carefully and thoroughly inspected by examining the affected paw and animal's general status. The health status parameter included paw volume, animal body weight, arthritic score and behavioural observations such as locomotor activity. The body weights of all the animals were recorded in grams on weekly basis by using single pan weighing balance. Body movement was measured by observing the time taken by individual animal to move two meter distance and statistical analysis is performed. The animals were sacrificed on day 41 to study the histology of the joint.

PARAMETERS:

1. Paw edema volume

Requirement:-

Plethysmometer

Mercury

Marker

Figure L. Plethysmometer

Procedure:-

Mercury Plethysmometer should be filled with mercury to a designated level. A mark was made on both the legs at lateral malleolus to facilitate uniform dipping and recording of paw volumes. The animal's paw was dipped into one column and the displacement of mercury was noted when the paw was dipped in mercury column up to a predetermined mark on the paw. The difference in paw volume indicates the degree of inflammation.

Evaluation:-

The hind paw volumes of all the animals were measured just before Freund's complete adjuvant injection on day 0 and thereafter at different time intervals (day 4, 13, 25, 40) using a plethysmometer instrument [12, 13 & 14]. The paw volume changes were calculated by subtracting initial paw volumes from the final paw volumes.

$$\text{Paw volume} = \frac{(Vc - Vo) - (Vi - Vo)}{(Vc - Vo)} \times 100$$

Where,

V_c = Paw volume after induction

V_o = Paw volume before induction

V_i= Paw volume after treatment

2. Arthritic score

Visual observation:-

The degree of arthritis was continuously monitored on day 0, 4, 13, 25 and 40 after injection of Freund's adjuvant. The arthritic score was monitored by set visual criteria and changes in the morphological feature of the arthritis like redness, swelling and erythema was noted and scoring was done.[60,61]

3. Locomotor activity

Instrument:-

Slab (Animal to travel a two meter distance)

Procedure:-

The locomotor activity of rats were recorded individually for each animal. Body movement was measured by observing the time taken by individual animal to move a certain distance. It was recorded on day 0, 4, 13, 25, 40 of the experiment.

Evaluation:-

The locomotor activity of an individual animal was measured and recorded.

4. Body weight

Apparatus:-

Weighing balance

Procedure:-

Measuring balance was calibrated and the mark was kept at zero. Each animal was placed in thebalance and the weight was recorded.

Evaluation:-

Body weight was recorded at different time intervals (day 0, 4, 13, 25, 40).Changes in body weight was calculated.

5.4 *INVITRO* ANTI-ARTHRITIC STUDY

1. Biochemical estimations

On day 41, after anesthesia(using ether vapor), cardiac puncture was done and a centrifuge tube was introduced to withdraw blood. Blood with and without anticoagulant was centrifuged for 15 min (3000 rpm) and the plasma and serum was collected. Total proteins such as albumin and globulin, Rheumatoid factor(R_f) and C-Reactive protein(CRP) levels were quantified.

Estimation of total protein:

Spectrophotometric method using erythrosine B dye was carried out for the determination of total proteins in blood plasma from rats.

Materials and reagents:

Bovine serum albumin (BSA-Sigma) solutions

3',3",5',5"-tetrabromophenolphthalein ethyl ester(TBPEE)

Ultraviolet and visible spectrophotometer

Procedure:

A 50 µl aliquot of blood plasma was transferred to a test tube and the volume was made up to 2.0 ml with distilled water. A 50 µl aliquot of this solution was transferred to a second test tube and the volume was adjusted to 2.0 ml with 0.2 mol L^{-1} of acetic acid. Standard curve was prepared by taking 0.0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 µl of standard solution of BSA (1.5 g L^{-1}), a calibration curve with the concentrations from 0.0 to 84.0 µg mL^{-1} was obtained, the volumes were adjusted to 2.0 ml with acetic acid (0.2 mol L^{-1}). After, in all tubes, 100 µl of TBPEE (0.005% m/v) was added, shaken and incubated at 37ºC for 10 minutes. The tubes were then cooled to room temperature and, after 30 minutes the absorbances at 610 nm were read against the blank (0.0 µg mL^{-1}).

2. Protein Denaturation Inhibition Study

Principle:

Denaturation is caused by the application of an external stress such as heat or by introducing chemical compounds such as strong acid or base, concentrated inorganic salt, an organic solvent. Proteins lose their tertiary structure and secondary structure thereby their biological function is lost. Protein denaturation is a well documented cause of inflammation.

Requirements:

- Bovine serum albumin (5% w/w aqueous solution)
- Plant extract
- Polyethylene glycol
- Phosphate buffer saline(pH 6.3)

Procedure:

The method of protein denaturation was conducted as per the method described by Mizushima and Kobayashi [62]. The reaction mixture consisted of0.45 ml of bovine serum albumin (5% w/w aqueous solution) and 0.05ml of ethanolic extracts of Mimosa pudica(Linn.) at variable concentrations ranging from 100 to 1000 µg in 10% v/v of polyethylene glycol. The pH adjustment was doneto 6.3 by adding 0.1N HCl and the samples were incubated at 37^0C for 20 min and then heated at 57˚C for 3min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The resulting turbidity was measured at 660 nm spectrophotometrically. For control tests, 0.05 ml distilled water was used instead of plant extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows. The control represents 100% protein denaturation. The results were compared with acetyl salicylic acid(positive control) in the present investigation.

$$\text{Percent Inhibition} = 100 - \frac{(O.D\ of\ test - O.D\ of\ product\ \ control\ )}{O.D\ of\ control} \times 100$$

3. Proteinase Inhibition Study

Principle:

Proteinase inhibitors play a major role in protecting the tissue against damage which is caused by proteinases and it has been implicated in the arthritic reactions. Neutral serine proteinases are present in the lysosomal granules of neutrophils which is considered to be a rich source of proteinases.

Requirements:

- Trypsin
- Tris–Hydrochloric acid buffer (pH 7.4)
- Casein
- Perchloric acid

Procedure:

The proteinase enzyme inhibitory assay was studied by the method described by Oyedapo et al. [63]. The reaction mixture contained 0.06 mg trypsin, 1.0 ml of 25 mM Tris–Hydrochloric acid buffer (pH 7.4) and 1.0 ml of ethanolic extracts of Mimosa pudica(Linn.) at variable concentrations ranging from 100 to 1000 µg in polyethylene glycol. The mixtures were incubated at 37^0 C for 5 minutes and then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for additional 20 minutes and 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged and absorbance of the supernatant was read at 280 nm against buffer as blank. The percentage of inhibition was calculated. The results were compared with acetyl salicylic acid (250 µg/ml) treated samples.

$$\text{Percent inhibition} = 100 - \frac{(O.D\ of\ test\ -O.D\ of\ product\ \ control\ )}{O.D\ of\ control} \text{ X } 100$$

OTHER METHODS

1. Histological analysis of ankle joints

The animals were sacrificed on day 41, the ankle joints were removed and preserved in

10% buffered formalin for 24 hours. It was followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 50 µm thickness. The sections were stained with hematoxylin and eosin H & E [64] and evaluated under light microscope for the presence of hyperplasia of synovium, pannus formation and destruction of joint space.

2. X-ray radiography

Rats were anaesthetized by intraperitoneal injection of 50mg/kg pentobarbitone sodium on day 41. Radiographs were taken with X-ray apparatus for lateral and mediolateral projection. The severity of the joint and bone deformation was blindly scored according to the extent of osteoporosis, joint spaces, osteophytes and joint structure [65,66] on a scale of 0-4

0 -No degenerative joint changes,

1. Slight soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes,
2. Low to moderate soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis,
3. Pronounced soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes,
4. Excess soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes.

STATISTICAL ANALYSIS

The data was analyzed in terms of Mean ± Standard error of Mean (SEM). For statistical analysis, multiple comparisons of data were made using one and two way analysis of variance (ANOVA) followed by Dunnet's test was used for post hoc analysis. Significance was statistically acceptable at a level of $P < 0.05$. Software program GraphPad Prism was used for all data analysis.[67]

VI. RESULTS

Acute toxicity results:

No toxic symptoms were observed after administration of different dose levels of extract up to maximum of 2000mg/kg p.o. according to OECD guideline 423; and in addition the higher dose of 2000mg/kg dose was administered to a group of animals. No symptoms or adverse events were identified. Hence, safe tolerable dose was used as therapeutic dose for further pharmacological study. From this experiment, the minimum and maximum therapeutic dose level of EMP extracts were studied as 200mg/kg and 400 mg/kg.

Invivo results

1. Paw edema volume

In FCA induced arthritis model, rats developed a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage, bone destruction and remodeling. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal[68].

The extract of *Mimosa pudicaLinn.*at 400 mg/kg inhibited rat paw edema which is comparable with standard drug methotrexate at40th day. The results of which are shown in Table 6 . The determination of rat paw swelling is apparently simple, sensitive and one of the quick procedures for evaluating the degree of inflammation and the therapeutic effects of drugs. The chronic inflammation involves the release of number of mediators like cytokines, GM-CSF, interferons and PGDF. These mediators are responsible for pain and destruction of bone, cartilage that can leads to severe disability.

2. Arthritic score

Picture 1 depicts the scoring of arthritis by set visual observation of the respective groups of animals after drug treatment and the results are as follows:

Group 1

Control

Normal paw with no macroscopic sign of arthritis

<u>Group 2</u>

Negative control

Gross deformity and inability to use the limb since multiple joints were inflamed and erythema progressively increased

<u>Group 3</u>

Standard, Methotrexate

Swelling and redness of joint is more when compared to the high dose of the extract

Group 4

LDMP

Moderate swelling and erythema of two joints after drug treatment

Group 5

HDMP

Reduced swelling and erythema of joints after drug treatment

3. Locomotor activity and body weight

As the incidence and severity of arthritis increased, there were changes in the body weights of the rats during the course of the experimental period. The loss of the body weight during arthritic condition was also supported by earlier observations[69], on alterations in the metabolic activities of diseased rats.

The body weight in standard group almost remained same during 40 days of study. In the low dose and high dose(200 mg/kg and 400 mg/kg) group of animals, body weight declined after 9 days of study and significant loss of weight was observed on 18^{th} and 25^{th} day. Methotrexate treatment did not produce any significant change in body weight. In non-treated group of rats, no significant change in behaviour was observed. During 9^{th} and 18^{th} day of study significant decrease in the movement of rats were noted both in plant extract and standard. However, on 40^{th}

day of study restoration of the normal movement was observed when compared with non-treated groups. The results of which are shown in table 7.

Invitro results

1. Effect of *M. Pudica* extracts on Biochemical parameters

The results in Table 8 shows that, in untreated animals (negative control group), serum levels of CRP and $R_f$significantly increased ($P < 0.001$) and total protein level significantly decreased ($P < 0.001$) compared to the parameters of animals of the healthy group. In animals treated with extracts or methotrexate, all biochemical parameters evaluated tend to return to normal values.

2. Protein Denaturation Inhibition Study

Anti–arthritic effect of EMP extractwas studied significantly by testing various in–vitro parameters. Table 9 depicts the inhibition of protein denaturation of different extracts. In the present investigation, all the three extracts inhibited the protein denaturation in a dose dependent manner. However, the EMP has got a higher inhibitory percentage of protein denaturation when compared ($p>0.05$) to thepositive control. At the concentrations of 400, 500, 800 and 1000µg/ml, the inhibitory percentage of EMP was significantly comparable to the positive control Acetyl salicylic acid used in this present investigation.

3. Protease Inhibition Study

The proteinase inhibitory activity of the EMP extracts were shown in Table 10. Both the EMP extract and the positive control acetyl salicylic acid exhibited a dose dependent anti–proteinase activity. At concentration of 800 µg/ml the EMP exhibited higher anti–proteinase activity of 75.18% when comparable ($p>0.05$) to the positive control which showed 73.69% of anti–proteinase activity at the same concentration. But at the maximum concentration of 1000µg/ml EMP showed 82.96% protease inhibition against the positive control acetyl salicylic acid which showed the inhibition of 85.90% at the same concentration.

Other methods results:

Analysis of histopathology of ankle joints

Histopathology of the ankle joint of healthy control rats revealed no inflammation, a few lymphocytes infiltration and no bone necrosis. A massive influx of inflammatory cells, cartilage destruction, proliferation of granulation tissue, lymphocytes infiltration and chronic inflammation was detected in arthritic control. In contrast to these pathological changes, animals having received ethanolic extracts of *M. Pudica* or indomethacin showed significant protection against necrosis of bones with low influx of inflammatory cells and minimal bone damage compared.

Histopathological analysis of ankle joints stained with H&E

Group 1

- Bone and cartilage between the jointsappeared normal.Synoviocytes surrounding the cartilage appeared normal
- No pannus formation and no inflammatory cells infiltration noticed

Group 2

- Severe pannus formation (chronic arthritis) surrounding the joints in which extensive proliferation of fibro vascular tissueor granulation tissue.
- Extensive accumulation of synovial fluids noticed in between pannus formation

Group 3

- Moderate pannus formation in which proliferation of fibro vascular tissue or granulation tissue.
- Also accumulation of synovial fluids in between pannus formation.

Group 4

- Bone structure appeared normal; no erosion noticed
- Cartilage/ synovialmembranes appeared normal
- Mild erosion noticed in the cartilage

Group 5

- Bone and cartilage surrounding the joints appeared normal
- Mild pannus formation in which proliferation of fibrous tissue noticed from the cartilage.

X-ray radiology of hind paws

Group 1

- Uninjected control group with no degenerative joint changes

Group 2

- Excess soft tissue volume, joint space, sub-chondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes

Group 3

- Moderate soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes.

Group 4

- Pronounced soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes.

Group 5

- Low to moderate soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes.

TABLES, GRAPHS AND FIGURES:

Table No. 1

Ash values of whole plant of Mimosa pudica Linn.

S. No	Type of ash	Percentage (W/W)
1.	Total Ash	5.257% w/w
2.	Acid Insoluble Ash	1.611% w/w
3.	Sulphated Ash	7.993% w/w

Table No. 2

Extractive values of whole plant of Mimosa pudica Linn.

S. No	Type of extractive value	Percentage
1.	Ethanol	23.758% w/w
2.	Water	18.964% w/w

Table No. 3

Nature of phytoconstituents present in the whole plant of Mimosa pudica Linn.

Phytoconstituents	Observation
Carbohydrates	+
Alkaloids	+
Proteins & Amino acids	+
Tannins & Phenolics	+
Flavonoids	+
Triterpenoids	-
Saponins	+
Fixed oils	+
Glycosides	-
Gums	-
Mucilage	+

(+)Indicates the presence of chemical constituents,

(-)Indicates the absence of chemical constituents

Table No. 4

Fluorescence analysis of the raw material and whole plant extract of Mimosa pudica Linn.

Sample		Reagent used	Visible	UV
Mimosa pudica (Linn.)raw powder (Whole plant)	1.	1(N) NaOH	Yellowish green	Green
	2.	1(N) NaOH in Alcohol	Yellowish green	Green
	3.	1(N) HCI	Light brown	Green
	4.	50% HNO_3	Orange	Green
50% ethanolic extract of Mimosa pudica (Linn.)(Whole plant)	1.	1(N) NaOH	Yellowish brown	Green
	2.	1(N) NaOH in Alcohol	Yellowish brown	Green
	3.	1(N) HCI	Light brown	Green
	4.	50% HNO_3	Orange	Green

Table No 5

R_f values of the TLC studies on the ethanolic extract of Mimosa Pudica Linn

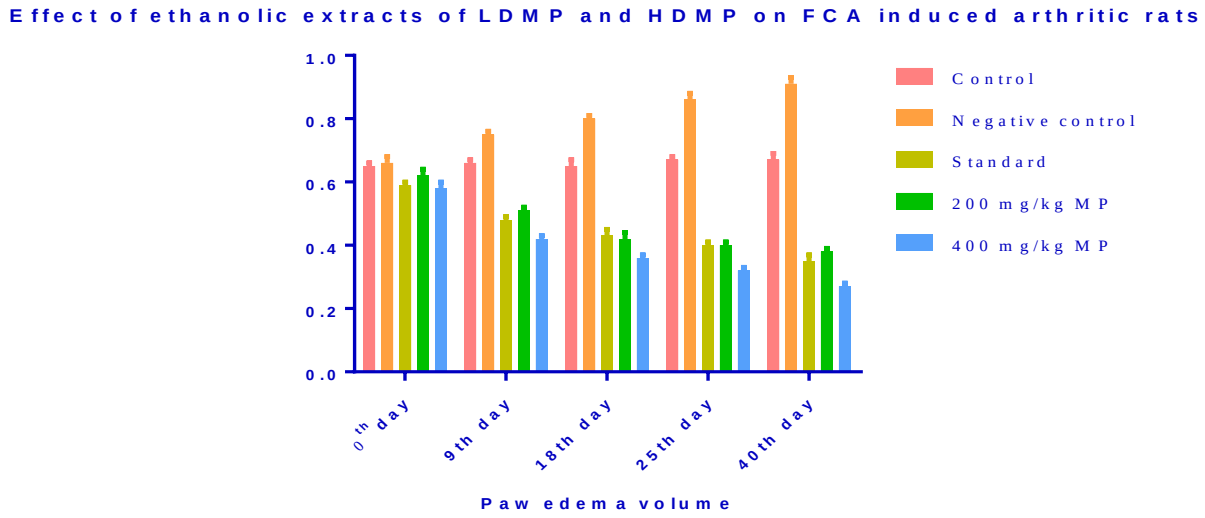
S. No	Extract(10 mg/ml)	Solvent System	TLC study for	R_f values
1.	Mother extract	Acetonitrile : methanol (8:2)	Alkaloid	0.54
2.	Alkaloidal fraction	Acetonitrile : methanol (8:2)	Alkaloid	0.51 0.80
3.	Methanolic fraction	Acetonitrile : methanol (8:2)	Alkaloid	0.56
4.	Ethyl acetate fraction	Acetonitrile : methanol (8:2)	Alkaloid	-

Table No 6

EFFECT OF MIMOSA PUDICA LINN EXTRACTS ON CHANGES IN PAW VOLUME INCFA INDUCED ARTHRITIS IN RATS

	Paw edema volume				
Group	**0^{th} day**	**9th day**	**18th day**	**25th day**	**40th day**
Control	0.65 ± 0.01	0.66 ± 0.01	0.65 ± 0.02	0.67 ± 0.01	0.67 ± 0.02
Negative control	0.66 ± 0.02	0.75 ± 0.01	0.80 ± 0.01	0.86 ± 0.02	0.91 ± 0.02
Standard	0.59 ± 0.01	0.48 ± 0.01	0.43 ± 0.02	0.40 ± 0.01	0.35 ± 0.02
200 mg/kg MP	0.62 ± 0.02	0.51 ± 0.01	0.42 ± 0.02	0.40 ± 0.01	0.38 ± 0.01*
400 mg/kg MP	0.58 ± 0.02	0.42 ± 0.01	0.36 ±0.01*	0.32 ±0.01**	0.27 ± 0.01*

Values are expressed in mean ± SEM, n = 6, *p<0.05 are significant compared to standard, LDMP(200 mg/kg); HDMP(400 mg/kg)



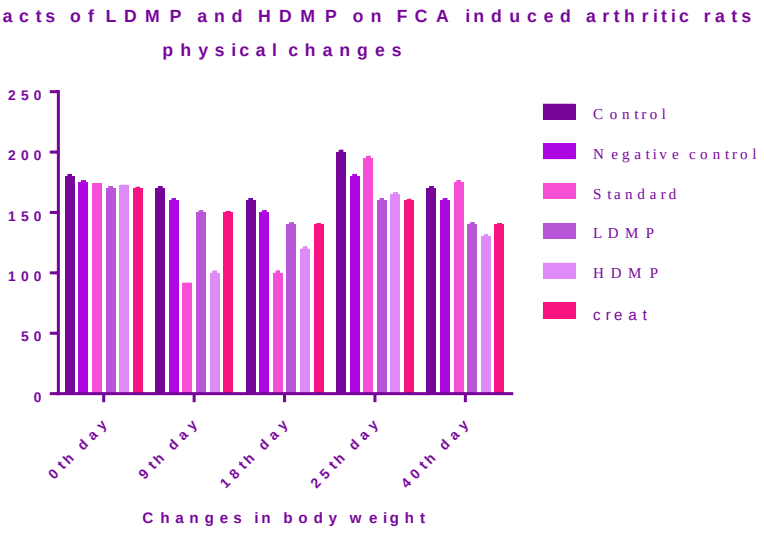
Graph I

Table No 7

Effect of ethanolic extracts of LDMP and HDMP on CFA induced arthritic rats showing changes in body weight and locomotor activity

Group	Physical and behavioural changes									
	0^{th} day		9^{th} day		18^{th} day		25^{th} day		40^{th} day	
	BW (gms)	M (sec)	BW (gms)	M (sec)	BW (gms)	M (sec)	BW (gms)	M (sec)	BW (gms)	M (sec)
Control	180±0.02	20	175±0.01	22	170±0.01	24	173±0.02	22	174±0.01	23
Negative control	170±0.01	20	160±0.01	30	150±0.02	35	100±0.02	50	92±0.02	55
Standard	170±0.01	20	160±0.02	30	140±0.01	32	130±0.01	32	175±0.01	25
LDMP	160±0.02	20	150±0.02	30	140±0.02	30	120±0.01	40	100±0.01	40
HDMP	200±0.02	20	180±0.02	25	160±0.02	25	165±0.01	30	195±0.01*	25

Values are expressed in mean ± SEM, n =6 ,*p < 0.05 are considered significant compared to standard. LDMP(200 mg/kg); HDMP(400 mg/kg); BW – Body weight; M - Movement



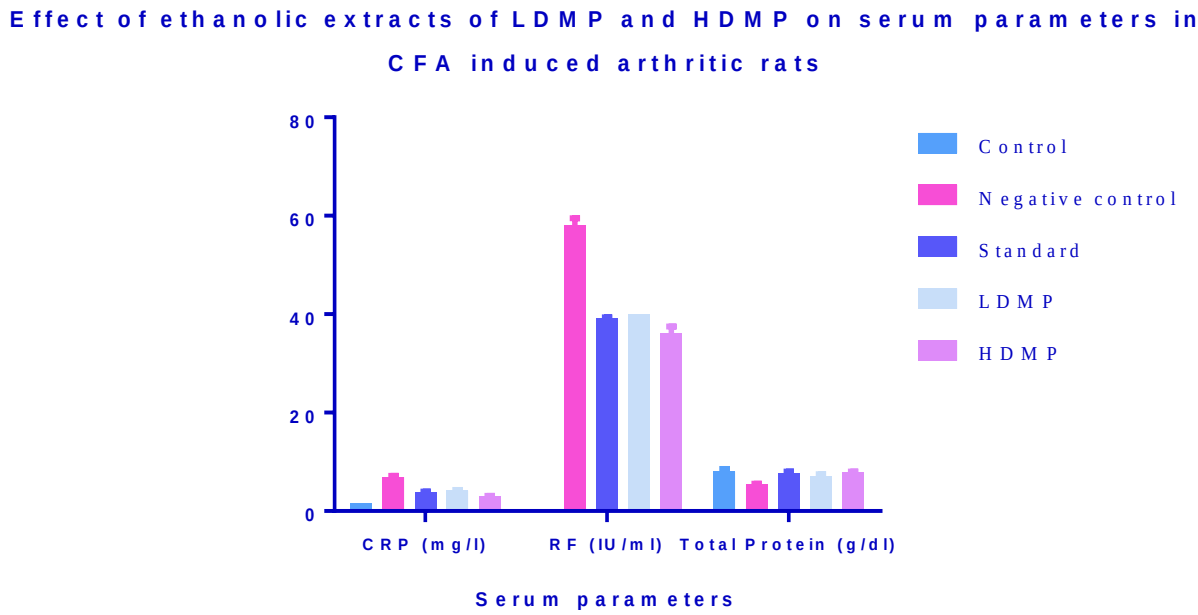
Graph II

Table No 8

Effect of ethanolic extracts of LDMP and HDMP on serum parameters in CFA induced arthritic rats

Group	CRP (mg/l)	R_f (IU/ml)	Total Protein (g/dl)
Control	1.65 ± 0.01	-	8.0 ± 0.54
Negative control	6.92 ± 0.22**	58.01 ± 1.50	5.4 ± 0.15*
Standard	3.68 ± 0.30**#	39.15 ± 0.23#	7.6 ± 0.42*
LDMP	4.12 ± 0.18**#	40.03 ± 0.02#	7.1 ± 0.43
HDMP	3.05 ± 0.05*#	36.01 ± 1.51#	7.8 ± 0.25#

Each value represents the mean ± SEM for ANOVA, n=6, *p<0.05, **p<0.001 when compared to healthy control ,#p<0.001 when compared to negative control.



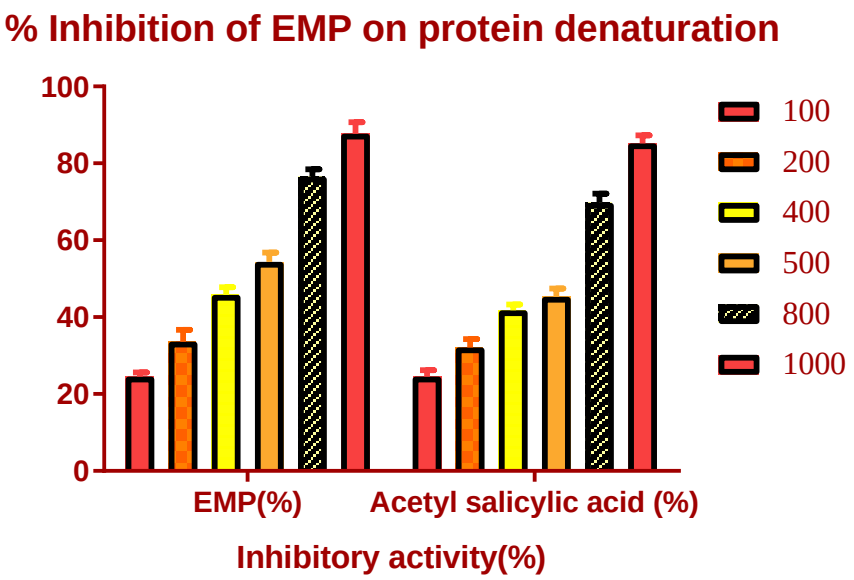
Graph III

Table No 9

Protein Denaturation Inhibition Study

Concentration (mg/ml)	Inhibitory activity of EMP	Inhibitory effect of Acetyl Salicylic Acid (%)
100	24.46 ± 1.12	24.45 ± 1.70
200	33.62 ± 3.08	32.14 ± 2.21
400	45.76 ± 1.98	41.77 ± 1.52
500	54.35 ± 2.37	45.33 ± 2.18
800	76.48 ± 1.92	69.71 ± 2.43*
1000	87.65 ± 3.01	85.17 ± 2.13*

Values are expressed in mean ± SD(n=6), *p<0.05. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test. Comparison between acetyl salicylic acid vs EMP



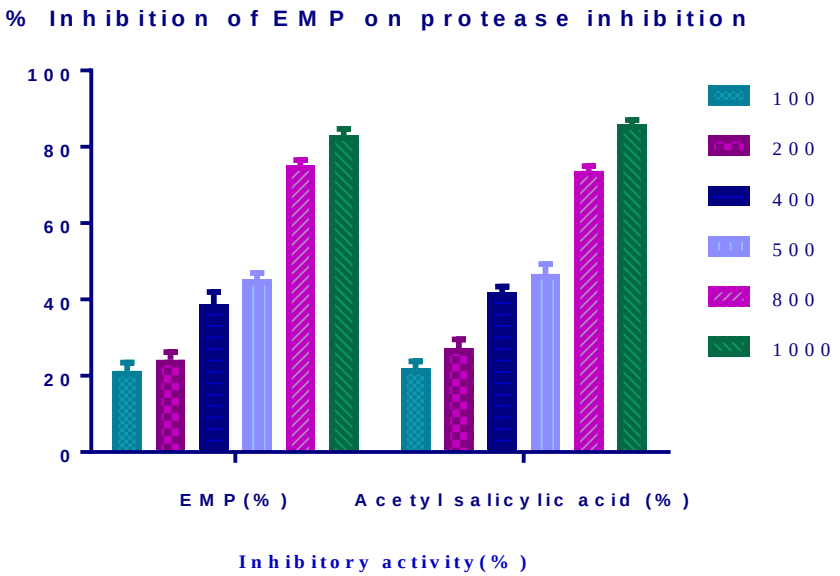
Graph IV

Table No 10

Protease Inhibition Study

Concentration (mg/ml)	Inhibitory activity of EMP	Inhibitory activity of Acetyl salicylic acid (%)
100	21.33 ± 2.18	21.88 ± 1.90
200	24.21 ± 2.00	27.21 ± 2.31
400	38.75 ± 3.12	41.75 ± 1.59
500	45.28 ± 1.65*	46.70 ± 2.58
800	75.18 ± 1.32*	73.69 ± 1.25
1000	82.96 ± 1.72	85.90 ± 1.10

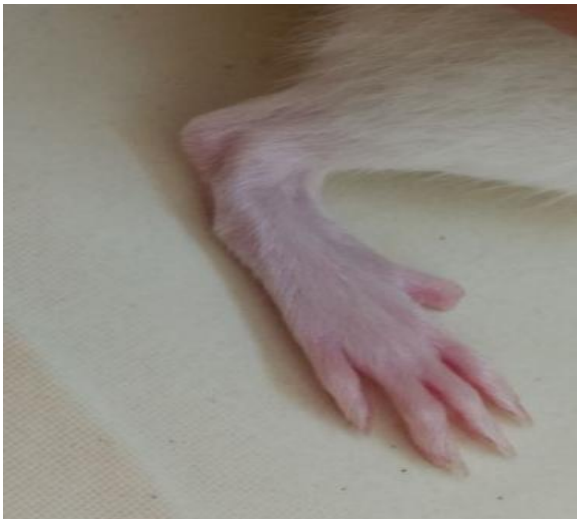
Values are expressed in mean ± SD(n=6), *p<0.05. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test. Comparison between acetyl salicylic acid vs EMP



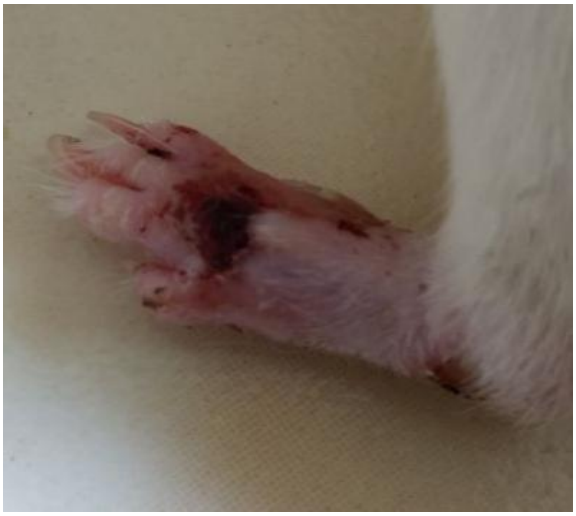
Graph V

PICTURES

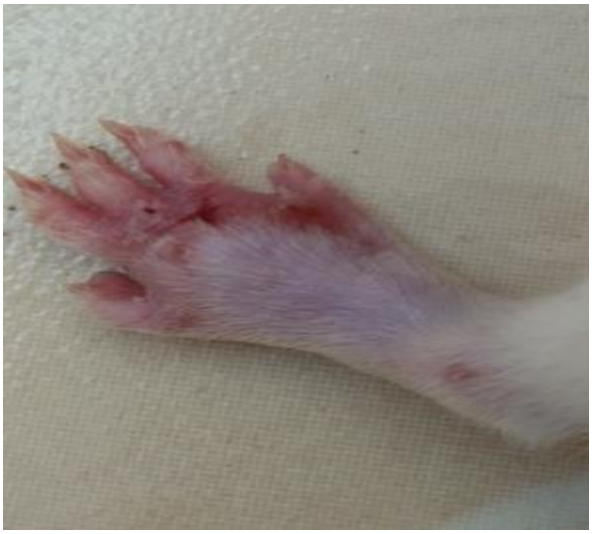
1. Arthritic score:



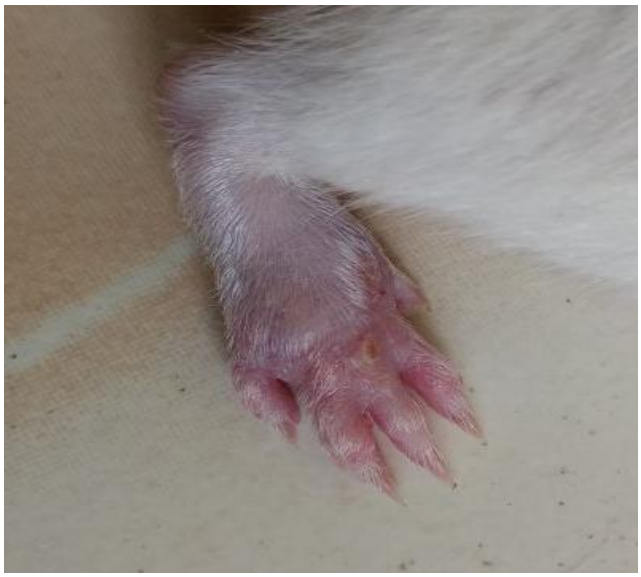
Group 1



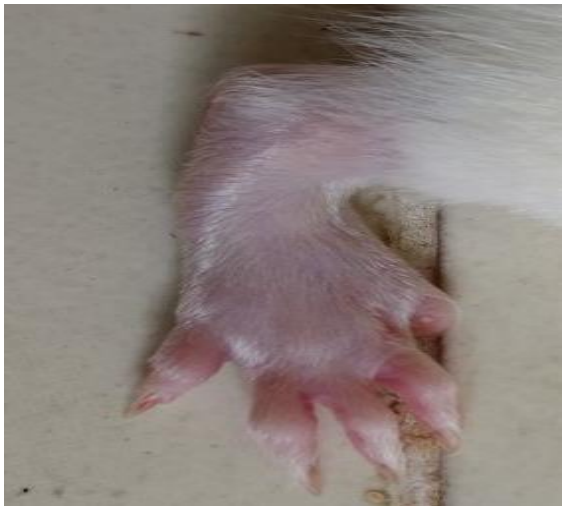
Group 2



Group 3

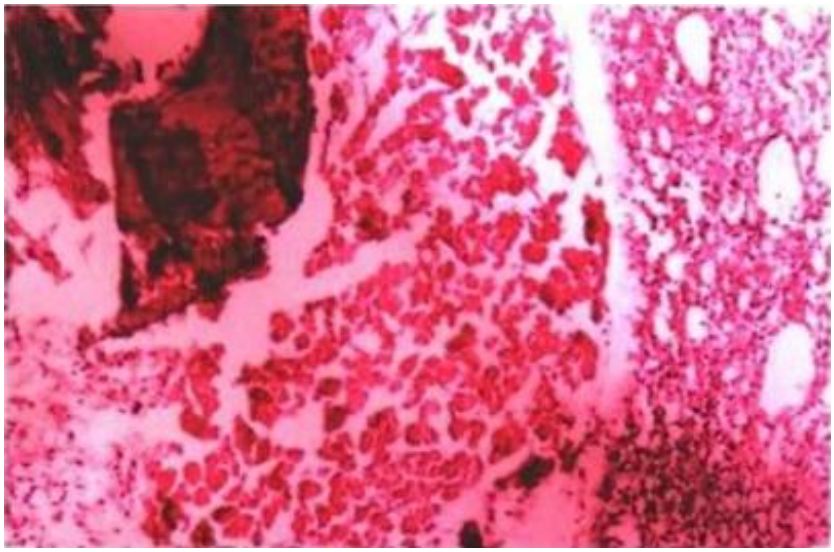


Group 4



Group 5

2. Analysis of histopathology of ankle joints



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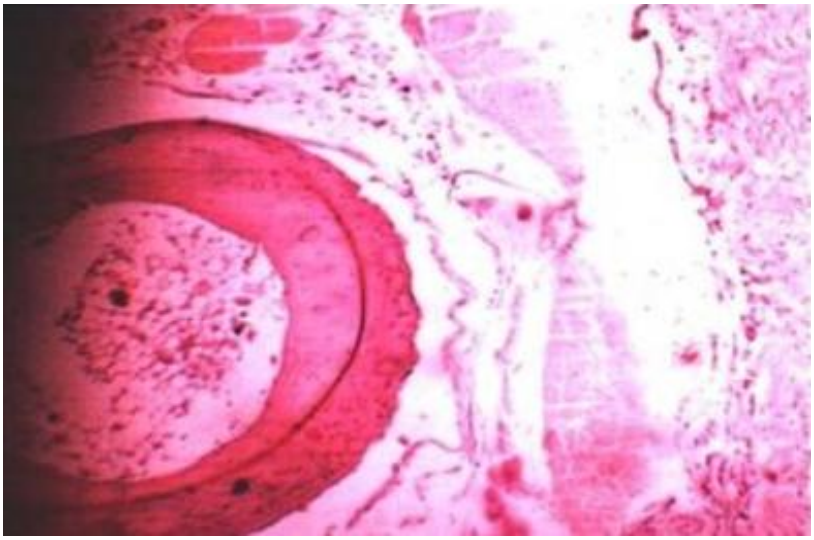

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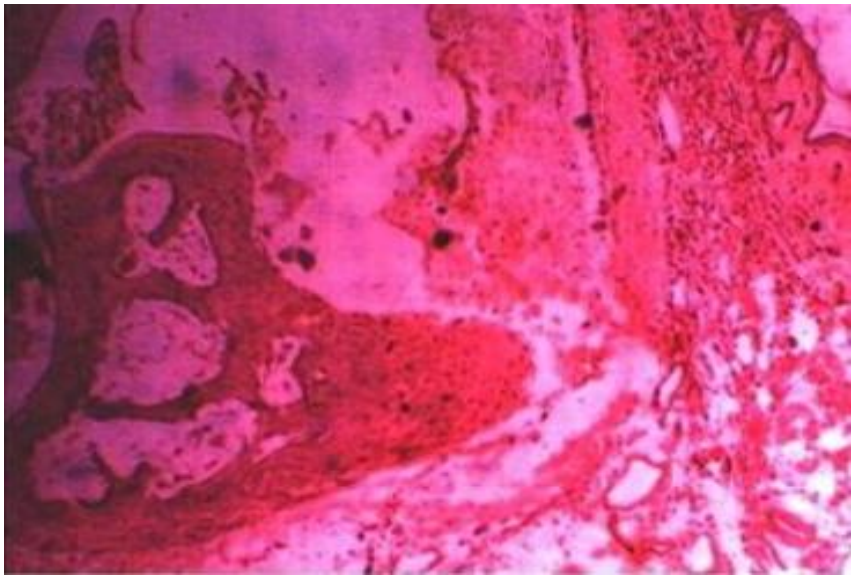

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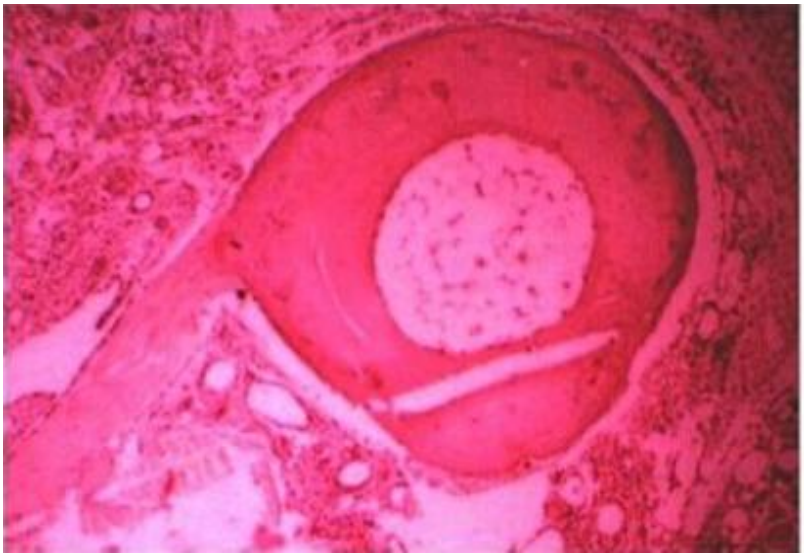

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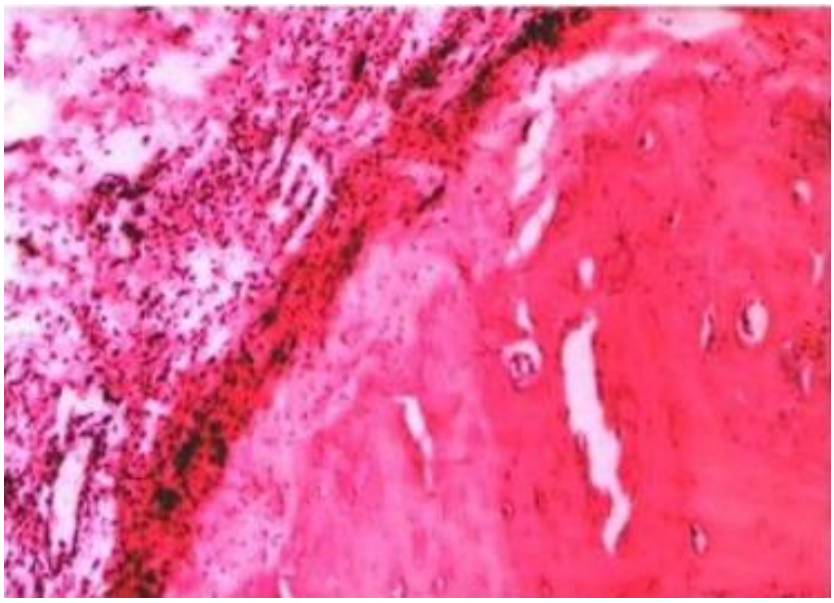
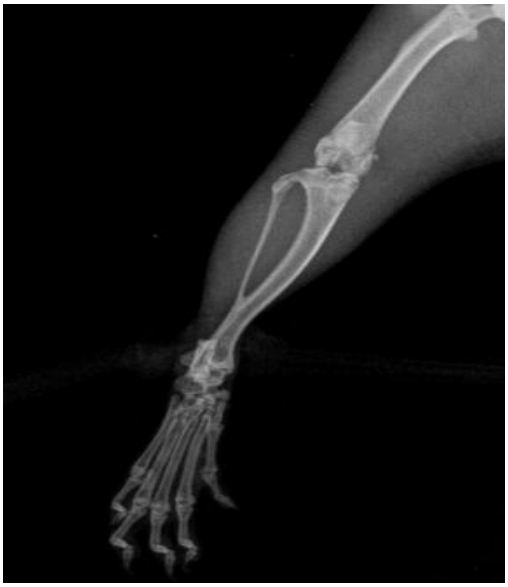
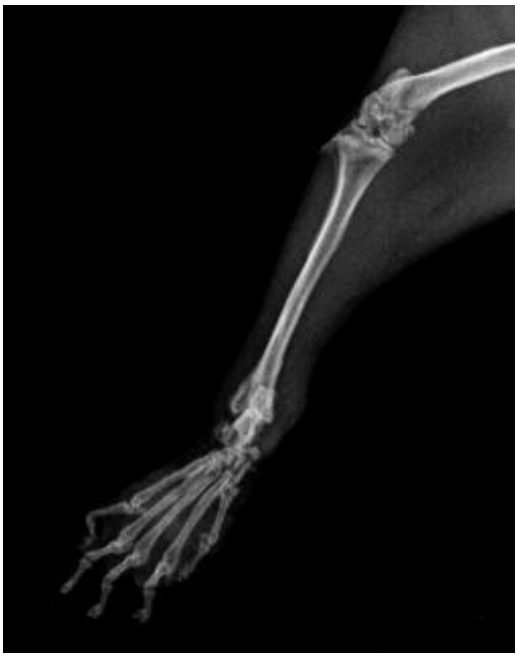


Group 5

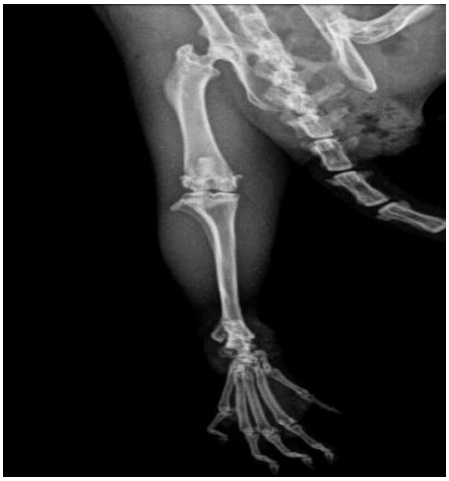
3. Radiography of ankle joints



Group 1



Group 2



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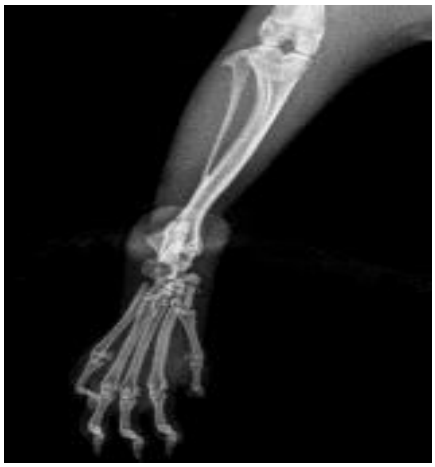

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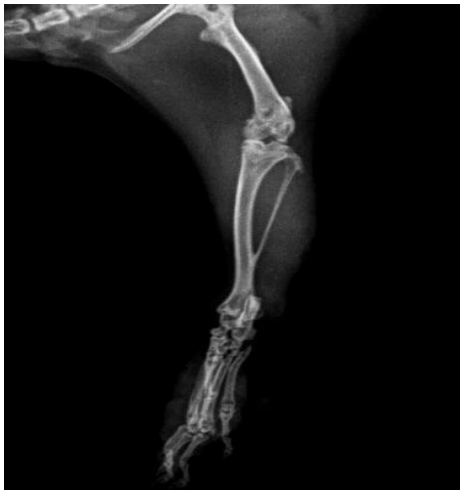

Group 5

VII. DISCUSSION

Rheumatoid arthritis is an inflammatory, autoimmune disorder which destroys it's own immune system. The immunologically mediated Complete Freund's adjuvant induced arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis[70].Complete Freund's adjuvant-induced arthritis is a model of chronic polyarthritis with features that resemble rheumatoid arthritis.

In Complete freund's adjuvant-induced arthritis model, rats developed a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling which have close similarities to human rheumatoid disease. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal. Also, the Complete Freund's adjuvant administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues.

Paw swelling is an index of measuring the anti-arthritic activity of Mimosa pudica Linn.at the dose level 200&400 mg/kg, p.o. Mimosa pudica administered groups showed marked reduction in paw volume when compared with the Negative control group (Group II). It was also found that there was significant weight loss when compared to standard[71]. The result of the present study also indicates that there is a close relationship between the extent of inflammation, loss of body weight and arthritic index. The arthritic scoring was done on the basis of visual observation where it can be seen that there is a marked reduction in the swelling and joint damage of the drug treated groups[72]. It was also noted that the high dose Mimosa pudica Linn. Extract proved its efficacy to reduce the inflammation of the paws. The locomotor activity of the animals were improved in Group 5 animals(HDMP) when compared to the standard animals.

Assessment of the levels of serum parameters provides an excellent and simple tool to measure the anti-arthritic activity of the target drug. The total protein such as albumin and globulin was comparatively equal in all the three groups such as control, plant extract and standard[73]. TheC-Reactive protein levels of the plant extract and standard was marginally equal

but higher than the control values. Rheumatoid factors are proteins produced by our immune system that can attack our own healthy cells of the body. When the levels rise, it might relate to some form of auto immune diseases. The R_f value of the high dose plant extract was excellent when compared to that standard.

Histopathology provides a noticeable morphological distinctiveness as a practical and unambiguous pathognomonic sign of Rheumatoid arthritis. The histopathological analysis identified the ability of the bones to re-form upon treatment with Mimosa pudica Linn. Bone structures re-calcified upon treatment with the Mimosa pudica Linn.dose dependently. The high dose of the plant extract exhibited good therapeutic potential from the study results and is therefore consistent with earlier findings that the ability of a drug to suppress inflammation, synovitis and protect a joint is desired in rheumatoid arthritis therapy.

Radiographic changes in Rheumatoid arthritis conditions are useful diagnostic measures which indicate the severity of the disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages (final stages) of arthritis[74]. The radiographic features of the rat joints in adjuvant induced arthritic model are shown in **Picture 3**. In Freund's adjuvant induced arthritic rat (group II), soft tissue swelling along with narrowing of the joint spaces was severe which implies the bony destruction in arthritic condition. The standard drug Methotrexate (0.75 mg/kg) treated groups have prevented this bony destruction and also there is moderate swelling of the joint. Similarly, according to histopathological studies, extracts of Mimosa pudica Linn.have shown significant prevention against bony destruction by showing less soft tissue swelling and narrowing of joint spaces in the 40 days of treatment when compared with Complete Freund's adjuvant (Negative control group).

VIII. SUMMARY

Indian sub-continent is a rich source of plant & animal wealth which is due to its varied geographical and agro climate regions. It is a well known fact that traditional system of medicines always played important role in meeting the global health care needs. Arthritis is one of the most common auto-immune inflammatory disorders, foremost cause of disability in western and developing countries. The presently available synthetic drugs in the market are not only economical exploitation but also associated with adverse effects. The synthetic drugs includes NSAIDS and DMARDS like Cyclophosphamide, intramuscular gold, sulfasalazine had the side effects of stomach ulcers, GIT bleeding, kidney, liver damage and hypertension. The given plant Mimosa pudica Linn.provides essential compounds with active principles, having no or minimum side effects holds prospect in future rheumatoid arthritis treatment. From the above review it should be manifest that there are many medicinal plants which exert anti-arthritic activity at a particular dose.

The preliminary phytochemical studies discovered the presence of various phytoconstituents.

Invivo study was performed with parameters such as paw edema volume, physical and behavioural changes and arthritic index and the extract possessed a significant effect on the inflammation and joint destruction.

The biochemical analysis were assessed by estimating the serum values which provided favourable effects.

Invitro study showed the effect of the plant extract on the percentage inhibition of protein denaturation and protease enzymes which gave marked responses.

Other methods such as histopathology and the radiographic X-ray analysis of the groups showed good results.

CONCLUSION

In conclusion, this study has verified that constituents of the plant suppressed the joint inflammation and destruction in adjuvant arthritic rats. We are confident that our data provide mechanistic evidence for anti-arthritic appliance of the plant as a promising candidate for novel therapeutic agent of Rheumatoid arthritis.

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ANNEXURE

INSTITUTE OF HERBAL SCIENCE
PLANT ANATOMY RESEARCH CENTRE

Prof.**P Jayaraman, Ph.D**
Director
Retd, Professor, Presidency College Chennai-5

AUTHENTICATION CERTIFICATE

Based upon the Organoleptic ✓ /macroscopic ✓ /~~microscopic~~ examination of fresh /market sample, it is certified that the specimen given by K. KEERTHI KANIMOZHI, M. Pharm Dept. of pharmacology, C.L. Baid Metha college of pharmacy. is identified as below:

Binomial: Mimosa pudica L.

Family: Mimosaceae

Synonym(s): -

Regional names: Tamil : Thotalvadi

Reg.No of the certificate: PARC/2017/3459

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(Prof.P.JAYARAMAN)

Prof.P.Jayaraman,Ph.D.
Director,
Institute of Herbal Botany
PLANT ANATOMY RESEARCH CENTRE,
No.4-II Street,Sakthi Nagar,
West Tambaram,Chennai-45.
Ph:044-22263236, Cell:9994136959
E-mail:herbalparc@yahoo.com

#4,2nd Street, Sakthi Nagar,
West Tambaram, Chennai-600 045
Ph:044-22263236,+919444385098
Email- herbalparc@yahoo.com