EFFICACY OF *GAULTHERIA FRAGRANTISSIMA* WALL. ON LYSOSOMAL DESTABILIZATION AND INFLAMMATION BY CFA INDUCED ARTHRITIS IN RAT

A Dissertation submitted to
THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY
CHENNAI - 600 032.

In partial fulfillment for the award of degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL BIOTECHNOLOGY

Submitted By
Reg. No: 26073743

Under the supervision of
Mr. I. Somasundaram, M.Pharm.
Department of Pharmaceutical Biotechnology

MARCH 2009

DEPARTMENT OF PHARMACEUTICAL BIOTECHNOLOGY
VEL'S COLLEGE OF PHARMACY
OLD PALLAVARAM,
CHENNAI - 600 117.

ACKNOWLEDGEMENT

First and foremost I express my sincere thanks to **GOD** for his blessings in completing my project.

I wish to express my profound thanks to my respective guide **Mr. I. SOMUSUNDARAM, M.Pharm.,** Assistant Professor; Department of Pharmaceutical Biotechnology; Vel's College of Pharmacy, for his continues source of inspiration, guidance given to me during my studies and project work.

I express my great source of respect to **Mr. T. S. SHANMUGARAJAN, M.Pharm., (PhD).,** Head of the Department; Department of Pharmaceutical Biotechnology; Vel's College of Pharmacy, for his encouragement, and extreme co operation throughout the course of my project work.

I wish to express my respect and gratitude to **Dr. V. RAVICHANDRAN, M.Pharm., Ph.D.,** Principal; Vel's College of Pharmacy, for lending me the all the required facilities in the college.

I wish to express my thanks to **Dr. V. GUNASEKARAN, M. Pharm, Ph.D.,** Vice Principal, Vel's College of Pharmacy, for his encouragement during my studies.

I wish to express my thanks to **Mr. V. LAVAKUMAR, M. Pharm.,** (PhD), Assistant Professor; Department of Pharmaceutical Biotechnology; Vel's college of Pharmacy, for his encouragement during my studies.

I wish to express my profound thanks to **Mr. V. Ravi Kumar, Mr. Rajesh Kumar Maurya, Mr. R. Rajasekar**, **Mr. Sunil, Ms. Meenu; Ms. Vasundara; Mr. Jai Kumar, Mr. Ashish, Mr. Narendra, Mr. Rishi, Mr. Rajesh Verma,** and **all my**

friends for their continues source of help, inspiration, and support given to me during my studies and my project work.

I wish to express my thanks to **Mr. R. SUMAN**, Lab Assistant, Department of Pharmaceutical Biotechnology, Vel's College of Pharmacy, for his support in arranging all the requirements and facilities.

Last but not least I would like to express my deep sense of gratitude to **my parents**; and **each and every member** of the family I belong to for their constant blessings, kindness and extreme support throughout my work.

ABBREVIATIONS

RA	=	Rheumatoid Arthritis
JA	=	Juvenile Arthritis
AS	=	Ankylosing Spondylitis
PAF	=	Platelet-Activating Factor
CD	=	Cluster Designation
CCP	=	Cyclic Citrullinated Protein
RF	=	Rheumatoid Factor
TNF-α	=	Tumor Necrosis Factor
IL	=	Interleukin
cAMP	=	Cyclic Adenosine Monophosphate
APC	=	Antigen-Presenting Cells
IFN	=	Interferon
RANKL	=	Receptor activator for nuclear kappa *k B* ligand
PUFA	=	Polyunsaturated Fatty Acids
OS	=	Oxidative Stress
ROS	=	Reactive Oxygen Species
NAG	=	N – Acetyl Glucosaminidase
CFA	=	Complete Freund's adjuvant
NSAIDs	=	Non steroidal Anti-Inflammatory Drugs
RER	=	Rough Endoplasmic Reticulum
NO	=	Nitric Oxide

ATP	=	Adenosine Tri-Phosphate
DNA	=	De-oxy Ribonucleic Acid
MHC	=	Major -Histocompatibility Complex
NADPH	=	Nicotinamide Adenide Dinucleotide Phosphate
SOD	=	Super oxide Dismutase
MDA	=	Malonaldialdehyde
OFR	=	Oxygen Free Radical

CONTENTS

INTRODUCTION

The Indian health care scene has inherited a large number of traditional practices, systems, and medicines as part of its total health care scenario, some of them more than 10000 years old. The earliest mention of the medicinal use of plants is to be found in the Rig-Veda which dates back as early as 3500 BC. Even though the modified systems of medicine include Ayurveda, Siddha, Unani and Tibetan; allopathic system medicine is the back bone of present medicine systems. It has been developed throughout meticulous scientific practices and fairly, effectual but still it have a tendency to show various type of adverse drug responses and have need of marvelous researches on this field. However on the other hand, the natural products, being the most significant source of therapeutics, is still at formative years and require more research's to make it more effective and safer as compared to the existing form.

Ayurveda, which is considered as an upaveda (or the supplementary Hymns designed for more detailed in structure of the humankind), is the very strong foundation stone of the ancient medical science of India. From the vast array of the materia medica of indigenous it is thought that investigation and research on medicinal plants might bring to the scientific world many useful remedies for the alleviation of human sufferings. Inspite of the remarkable achievements of modern medicine and medical research, these ancient systems continue to be a major component, "effectively" used in the control or alleviation of diseases.

Based on the strong traditional knowledge on the use of plants as therapeutic agents, a rational approach is being developed to use medicinal plants as lead for the discovery of active molecules, with one of the largest reservoirs of bio-resources. It is imperative that India develops a concerted, integrated, structural, and modern approach in this area and gains a competitive edge in the international market place for the discovery and development of Natural drugs for a variety of diseases for which currently appropriate remedies are not available. All over World, several pharmaceutical companies and research organizations are concentrating on the cosmic untapped potential of herbals as potential drugs.

Indian systems of Medicine mainly focus on herbal medicines for prevention of diseases, to increase the prolonged existence and for heal of chronic diseases. India has very flourishing resources of medicinal plants due to wide bio- diversity. There has been a prominent raise in utilization of herbal drugs in both developing and the developed countries due there natural origin and minimal or no side effect.

Several important constituents of plant origin such as alkaloids, glycosides, terpenoids, flavonoids, antibiotics etc. have been isolated and used as the therapeutic purposes. It is very important that India develops a concerted, integrated, structural, and modern approach in research area and gains a competitive edge in the international market place for the discovery and development of Natural drugs for a variety of diseases for which currently appropriate remedies are not available for cure them.

According to WHO, traditional medicine is defined as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manuals techniques and exercises applied as singularly or in combination to treat, diagnose and prevent illnesses or maintain well being. The term traditional medicine is applied due to the fact that they had their origin in the remote past and most of these are still practiced almost in the same approach as have been in the ancient times, maintaining the tradition. The basic principles involved in traditional system of medicine are that these are holistic in nature and rather than treating in isolation, they are believed to eradicate the root cause of the disease.

Arthritis

Arthritis is a group of conditions that affects the health of the joints in the body particularly the bones of the hands and feet, which causes pain, swelling, stiffness, redness, and heat. Arthritis as the name suggest, means “joint inflammation” from (arthro - joints + itis - inflammation; plural: arthritis). Nearly 100 different types of arthritis has already been reported which affects different areas of the body and all age groups. Arthritis is the leading cause of disability in humans mostly over 55 years of age with certain exceptional cases where the patient may be obese.

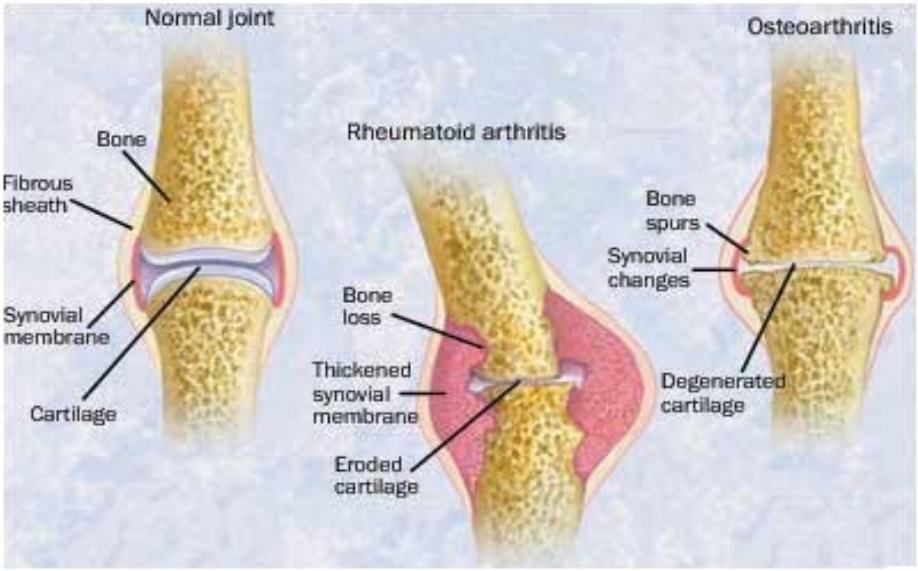


Figure-1: Comparison of Normal and Arthritic joint

Common types of Arthritis

- *Rheumatoid arthritis*: - Rheumatoid arthritis (RA) is a state of chronic, progressive, inflammatory, multisystem, autoimmune disorder, commonly polyarticular, which attacks multiple joints especially smaller joints of the hands and feet.

- *Osteoarthritis*: - Osteoarthritis also called "wear and tear" arthritis involves the gradual deterioration of cartilage usually in the larger, weight-bearing joints, which ultimately leads to trauma of joint.

Other types of Arthritis

- *Juvenile arthritis*: - Juvenile arthritis (JA), also called as Juvenile Rheumatoid Arthritis, refers to numerous childhood conditions that involve joint pain and inflammation, usually persisting for more than six weeks.
- *Ankylosing spondylitis*: - Ankylosing spondylitis (AS) is a form of Spondyloarthritis, which primarily causes eventual fusion of spine. Complete fusion however results in complete rigidity of the spine (bamboo spine).
- *Septic arthritis*: - Septic arthritis, also known as infectious arthritis, represents a direct invasion of joint space by various microorganisms, including bacteria, viruses, mycobacteria, and fungi.

Rheumatoid arthritis

Rheumatoid arthritis is an inflammatory autoimmune disease that is characterized by the destruction of joint cartilage and inflammation of the synovium (joint fluid) It affects particularly the joints of the ankles and toes, which causes soft tissue swellings (Blake & Lunce, 1985), and synovitis, which is the hallmark of an inflammatory arthritis such as rheumatoid arthritis. Although rheumatoid arthritis most often affects the joints, it can also affect many organs and body systems in the body. Systemic complications include damage to various organs (lungs, pleura, pericardium, myocardium, eyes, and central nervous system) due to inflammatory reactions. As the disease progresses, osteoporosis of the contiguous bone, destruction of joint cartilage, bone resorption, and displacement by ankylosis occur.

Inflammation as in the case of rheumatoid arthritis has three different phases:

- The first phase shows increase of vascular permeability.
- The second phase involves exudation of fluid from the blood into the tissues.
- The third phase leads to granuloma formation (Jasin, 2005).

Accordingly, anti-inflammatory test is divided into those measuring acute inflammation, sub acute inflammation, and chronic repair processes. These studies are aimed to find out new drugs against polyarthritis and other rheumatic diseases. An array of physiological substances, called autacoids, are involved in the process of inflammation and repair. Some of these include histamine, serotonin, bradykinin, substances P, group of Eicosanoids (prostaglandins, thromboxanes and leucotrienes), and the platelet-activating factor (PAF) as well as few cytokines and lymphokines. Rheumatoid Arthritis is 2 to 3 times more common in women than in men, and generally affects people between the ages of 20 and 50. Odema marks the onset of Rheumatoid arthritis exhibiting the early stages, which is manifested by joint swelling and pain.

Signs and Symptoms of Arthritis

Rheumatoid arthritis usually affects the joints of the hands (such as the knuckle joints), wrists, elbows, knees, ankles, and/or feet. The larger joints, such as the shoulders, hips, and jaw may be affected.

The usual joint symptoms include the following: -

Stiffness: The joint does not move as well as it once did. Its range of motion (the extent to which the appendage of the joint, such as the arm, leg, or finger, can move in different directions) may be reduced.

- **Inflammation:** Redness, tenderness, and warmth are the hallmarks of inflammation.
- **Swelling:** The area around the affected joint is swollen and puffy.
- **Nodules:** These hard bumps appear on or near the joint. They often are found near the elbows. They are most noticeable on the part of the joint that juts out when the joint is flexed.
- **Pain:** Pain in rheumatoid arthritis can come from inflammation or swelling of the joint and surrounding tissues or from working the joint too hard.

Pathogenesis of Rheumatoid arthritis

Rheumatoid arthritis mostly affects joints where cartilage overlies the bone and with a joint cavity lined by synovial membrane that contains synovial fluid. There are two popular theories that explain the pathogenesis of rheumatoid arthritis (RA). The first theory states that the T cell interacts with an unidentified antigen and acts as the primary cell responsible for initiating the disease as well as for driving the chronic inflammatory process.

This theory is based upon the known association of RA with class II major histocompatability antigens, the large number of CD4+ T cells and skewed T cell receptor gene usage in the RA synovium.

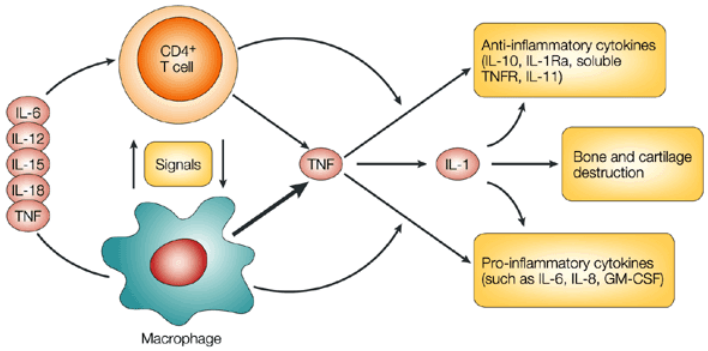


Figure-2: Role of Macrophages and CD4+ T cell in pathogenesis of Arthritis

The second theory holds that, while T cells may be important in initiating the disease, chronic inflammation is self-perpetuated by macrophages and fibroblasts in a T-cell independent manner. This theory is based upon the relative absence of activated T cells phenotypes in chronic RA and the preponderance of activated macrophage and fibroblast phenotypes.

Macrophages play the most important role in secretion of RF, antibodies, cytokines, anti CCP. This leads to change in synovium of RA due to infiltration of macrophages, T cell and B cell as well pannus formation that leads to, erode the articular cartilage and bone and finally destruction of joint occurs.

Cytokines have a wide range of activity in rheumatoid joint and it shows painful inflammatory signs and symptoms of RA. It is believed that they play a vital role in both physiological and pathological regulation of bone. These pro-inflammatory cytokines and proteolytic enzyme act as mediators in RA and it plays an important role in pannus formation, proliferation of synovial cells and destruction of bone and cartilage. Cytokines can induce oxidative stress while oxidative stress can increase levels of pro-inflammatory cytokines. It is well known that leucocytes produce pro-inflammatory cytokines such as TNF-α and IL-6, which play important roles in both RA in humans and AA in rats (Silva *et al*., 2000).

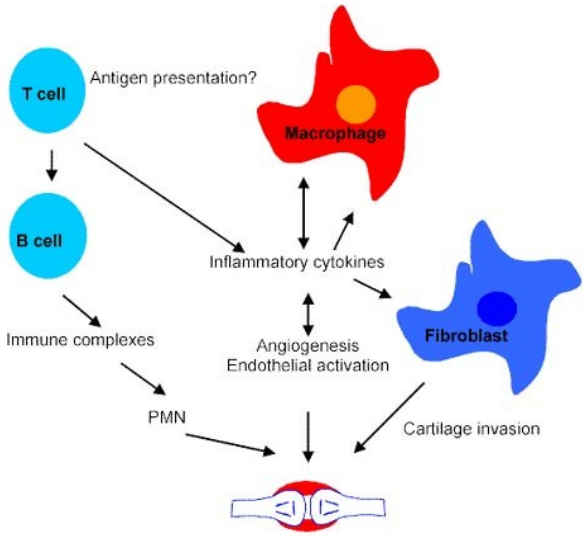


Figure-3: Role of T cell and B cells in bone damage

Cytokines such as TNF-α is a major important factor of RA and mostly formed by macrophages in the synovial membrane and at the cartilage–pannus junction. Irregular expression of TNF-α is the major cause for the development of chronic arthritis.

IL-6 also is a type of cytokine, which plays important role in acute arthritic conditions. An elevated level of this cytokine in synovial fluid is directly correlated with degree of radiological joint damage and thus it promotes the formation of osteoclasts. IL-6 is significantly produced by synovial fibroblasts and insignificantly by macrophages (Wood NC *et al*., 1992). It helps to protect the cartilage in acute conditions and it encourages the bone formation in chronic condition.

The RA synovial tissues contribute to cartilage loss by two principal mechanisms. The first is indirect and involves the effects of cytokines and other

mediators released from the inflamed synovium that produces dysregulation of chondrocyte function. TNF-α is a synovial factors that function in this manner. The second mechanism by which RA synovial tissues adversely affect cartilage remodelling is direct and involves products, in addition to cytokines; produced by the RA synovium that have the capacity to degrade the articular cartilage matrix.

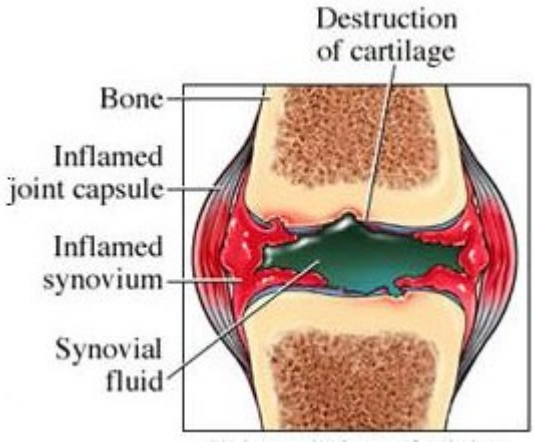
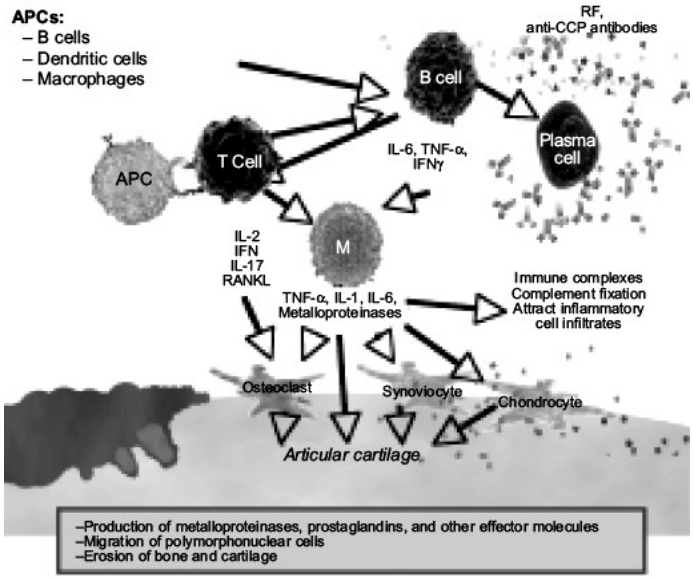


Figure-4: Enlarged view of arthritic joint

It has been reported that, T cells play an important role in the pathogenesis of RA (Kong *et al*., 1999). The generation of reactive oxygen species occurs when pathogens (bacteria) or immune complexes are ingested by phagocytic cells that form an oxidative burst to kill the invading pathogens (Lotz, 2003). Free radical such as reactive oxygen species and reactive nitrogen species both are free radicals, which involved in stimulation as well as degeneration of synovium (Jaswal *et al*., 2003). Antigen-presenting cells (APC) such as dendritic cells, macrophages, polymorphonuclear leucocytes, and T cell interrelate with plasma cell and B cell. This oxidative burst is mediated by the NADPH oxidase system and results in a marked increase in oxygen consumption and the production of superoxide ($O_2^{-\bullet}$) and this is the first line of defense against pathogens.

Superoxide plays a pivotal role in activation of the T-cell and T-cell mediated IL-2 and IFN-γ secretion (Smolen *et al*., 2007) which in turn increase the proliferation the T-cell. On the other hand it is also mediated by another strong reactive species H_2O_2 generated from superoxide (Dröge, 2002) and H_2O_2 activates the transcription factor NF-kappa B (Dumont *et al*., 1999) which regulates the expression of several cytokine

genes including IL-2 and TNF-α resulting the inflammation of joints (Baeuerle & Henkel, 1994).



APC = antigen-presenting cell; CCP = cyclic citrullinated peptide; IFNγ = interferon γ; IL = interleukin; MΦ = macrophage; RANKL = receptor activator for nuclear factor κ B ligand; RA = rheumatoid arthritis; RF = rheumatoid factor; TNF-α = tumor necrosis factor-α.

Figure-5: Pathogenesis of Arthritis

It is reported that mostly synovial cells are derivative of T cell. When antigenic peptides binds to class II MHC molecules retrieve the binding of activated macrophage or oxidative burst to the T-cell receptor. As a result of which T-cell will get active and undergoes a final activation by binding of co stimulatory receptor CD-28 on T-cell and APC surface molecules (ligand) that is CD-80 and CD-86 (Frauwirth & Thompson, 2002). Strong activation of this co stimulatory receptor CD-28 leads to oxidative stress and its helps significantly decrease in intracellular glutathione redox state as well as increase the production of H_2O_2 causes further T-cell differention and proliferation (Los *et al*., 1995). B cell acts as antigen receptor due to presence of surface immunoglobin. Strong evidence reported that B cell makes a major contribution to the inflammatory process. T-H_1 is a subset of T cell and it stimulates the activation of B-lymphocyte, fibroblast and osteoclast (Bombara *et al*., 1993). This co stimulation of B-cell by T-cell through cell surface molecule on B-cell leads to differentiate into plasma B-cell which secrets autoantibodies (Steiner & Smolen, 2006).

The high level of RF is a major immunologic abnormality in RA which forms immune complexes that reinforce the production of proinflammatory cytokines such as TNF-α and IL-6 via complement and Fc-receptor activation (Debets *et al*., 1988) and finally these proinflammatory cytokines can stimulate RANKL, which enhanced the osteoclast differentiation and bone erosion. Another strong ROS hydroxyl radical ($OH^{\cdot}$) is formed from H_2O_2 by Fenton reaction (Pillinger & Abramson, 1995) also peroxidize the polyunsaturated fatty acid (PUFA) of cell membrane of synovium which plays an important role in membrane destabilization.

Role of Oxidative Stress in RA

Oxidative stress occurs when there is an imbalance between generation of reactive oxygen species and inadequate antioxidant defense systems. Oxidative Stress (OS) is a general term used to describe the steady state level of oxidative damage in a cell, tissue, and disruption of macromolecular structure caused by the reactive oxygen species (ROS) (Davies, 2000). Oxidative stress plays an important role in the pathogenesis of RA.

Oxidative stress constitutes a serious pathophysiological factor for a wide variety of connective tissue disorders such as RA. In recent years, increasing attention has been given to the role of reactive oxygen metabolites in the pathogenesis of RA. Increased activity of free radicals, the unstable molecules associated with cell damage, is theorized to underlie the mucosal injury commonly seen in the various inflammatory diseases as RA (Lunec J *et al*., 1981).

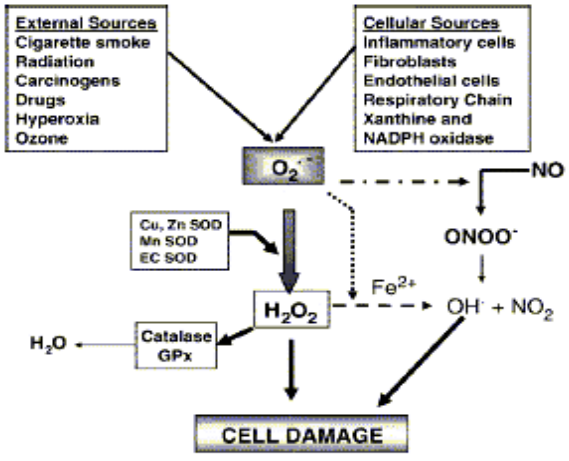


Figure-6: Sources of Cell damage

There are many different sources by which the reactive oxygen species are generated. ROS are the root cause of oxidative stress most of which come from the endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria (Shouval & Elazar, 2007) or the detoxification reactions involving the liver cytochrome P-450 enzyme system. Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to ionizing radiation, and bacterial, fungal, or viral infections. ROS and other oxidants have been shown to be formed even in the normal physiological process (Abuja PM *et al*., 2001). It is known that activated inflammatory cells lead to ROS production in RA a systemic autoimmune disease (Fox DA *et al* 2005). Increased reactive oxygen species (ROS) and other free radicals play an important role in the inflammatory process and contribute to tissue destruction can initiate lipid peroxidation and DNA damage leading to mutagenesis, carcinogenesis and cell death.

Pathogenic mechanism of chronic inflammation is associated with increased the production of ROS and free radicals (superoxide anion and It hydrogen peroxide). Evidence suggested that increased oxidative stress as well as defective antioxidant status act as mediators in the pathology of rheumatoid arthritis (De Leo ME *et al*., 2002). The study showed raised levels of Malondialdehyde (MDA) and low levels of endogenous antioxidants in patients of rheumatoid arthritis. It has been reported that, the level of Plasma catalase is significantly lower in arthritic patients (Kamanli A *et al*., 2004) and decrease the activity of glutathione reductase in the synovial fluids in-patient of rheumatoid arthritis (Bazzichi L *et al*., 2002).

The determinants of oxidative stress are regulated by an individual's unique hereditary factors, as well as his/her environment and characteristic lifestyle. Unfortunately, under the present day life-style conditions many people run an abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions.

Cytoskeletal damage: Reactive oxygen species are also known to cooperate with cytoskeletal elements and interfere with mitochondrial aerobic phosphorylation; moreover, ATP depletion occurs (Buettner & Schafer, 2000).

Cartilage/ collagen damage: Free radical enhanced the damage of cellular elements in cartilage directly and damage components of the extracellular matrix either directly or indirectly by upregulating mediators of matrix degradation (Henrotin YE *et al*., 2003). Reactive oxygen species Damage chondrocyte responses to growth factors and relocation to sites of cartilage injury; Reactive nitrogen species such as NO interfere with interactions between chondrocytes and the extracellular matrix (Clancy RM *et al*., 1997). NO may also increase chondrocyte apoptosis.

LYSOSOME

Lysosomes discovered by the Belgian cytologist Christian de Duve in 1949 are membrane bound organelles that contain several digestive enzymes. Lysosome obtained the name from Greek meanings (lysis – destruction; soma - body). It is also known as suicidal bags by cell biologists due to their vital role in autolysis. Lysosome fuses with vacuole, releases the lysosomal enzymes in to vacuole, and thereby helps in digestion of contents of the vacuole. Lysosomal enzymes are mostly active at acidic pH of 4.5. It is reported that interior portion of the lysosome is far more acidic (4.8) than that of the cytosol (7.2).

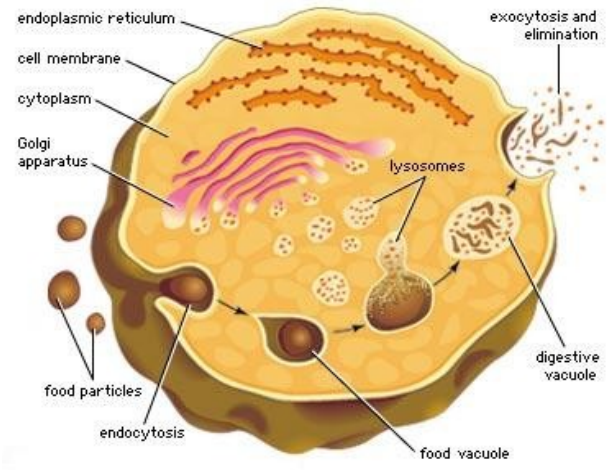


Figure-7: Basic Cellular Structures

Other functions include digesting foreign bacteria (or other forms of waste) that invade a cell and helping repair damage to the plasma membrane by serving as a membrane patch, sealing the wound. In the past, lysosomes were thought to kill cells that were no longer wanted, such as those in the tails of tadpoles or in the web from the

fingers of a 3- to 6-month-old fetus. While lysosomes digest some materials in this process, it is actually accomplished through programmed cell death, called apoptosis.

Functions

They are used for digestion of macromolecules; from phagocytosis (ingestion of other dying cells or larger extracellular material), endocytosis (where receptor proteins are recycled from the cell surface), and autophagy. Autophagy may also lead to autophagic cell death, a form of programmed self-destruction, or autolysis, of the cell, which means that the cell is digesting itself.

Lysosomal enzymes

Lysosomal enzymes are synthesized in the cytosol and endoplasmic reticulum and it receives a tag of mannose-6- phosphate that helps the enzymes to target itself to the lysosomes. Few of them are explained below.

Acid phosphatase: Acid phosphatase comes under the category of acid hydrolases and it consists of subunits that polymerizes at an acidic pH, thus it shows high enzymatic activity in specific granules of eosinophils (Bass DA *et al*., 1981, Weller PF *et al* 1983). It is mainly involved in the catabolism of glycoprotein, glycolipid, and glycosaminoglycans.

Cathepsin-D: Cathepsin-D is an aspartic endo-protease and it is widely distributed in lysosome (Barret AJ *et al*., 1970). Cathepsin-D plays a pivotal role in degradation of protein-degrading enzyme and protein in lysosome at acidic pH. Reports suggested that cathepsin-D also participate in activation of biologically active proteins in pre lysosomal compartments of specialized cells (Diment S *et al*., 1989). Cathepsin-D is synthesized on the rough endoplasmic reticulum (RER) as a pre-pro-enzyme that undergoes several proteolytic cleavages during biosynthesis to produce the mature form (Richo G *et al*., 1994).

β Glucuronidase: β Glucuronidase is mostly localized in lysosome and some amount is present in the endoplasmic reticulum. It catalyses the hydrolysis of glucuronides and it shows some idiosyncrasy function. It half-life is very short and it clears itself from the plasma by the liver Kupffer cells. β Glucuronidase is shown to be a sensitive marker of lysosomal integrity (Ravichandran LV *et al*., 1990).

N - Acetylglucosaminidase (NAG): NAG, a lysosomal enzyme ubiquitous to cell and it is normally present in the cells as well as human plasma. It has been report that elevated levels of NAG found in several inflammatory condition and its leads to alter the metabolism of different connective tissue constituents such as cartilage, glycoprotein, and glyaminoglycans(Price RG *et al*.,1972).

Arthritis Models: Several types of models are available for arthritis in rats. Some of the following models as mention below.

Pristane-induced arthritis in rat: It is also a type of animal model for rheumatoid arthritis induced with well-defined synthetic immunological adjuvant oil, pristine. Pristane induced arthritis developed after two week with a single intradermal injection of 150 micro liters of pristine , as well as the rat suffered sever and chronic arthritis .The inflammation was restricted to the joint sand involved pannus formation, major histocompatibility complex (MHC) Class II expression and T-lymphocyte infiltration (Crofford & Wilder, 1997).

Collagen type II induced arthritic: It is an extensively studied animal model of RA. It shows various pathological and an immunological features of human RA. CIA is primarily an autoimmune disease of joints, requiring both T and B cell immunity to autologous type II collagen (CII) for appearance of disease (Wilder *et al*., 1999).

Adjuvant induced arthritis: This type of arthritis induced by an intradermal injection, of heat killed Mycobacterium tuberculosis in IFA at the base of the tail, results in destructive arthritis within 14 days in susceptible DA or LEW inbred rat strains. AIA can also be induced with cell walls from other bacterial types in IFA, although the arthritogenicity varies. Its shows elevated level of cytokines due to increase the synthesis of tumor necrosis factor a (TNF-α), interleukin 1 (IL-1) and IL-6 is detected as early as day four after adjuvant injection. Granulocytes and auto reactive CD41 cells play major roles in the disease. Humoral immune mechanisms appear not to contribute to the disease process. This unique rat disease model represents a systemic process that involves not only the joints but also the gastrointestinal and genitourinary tracts, the skin and the eyes (Wilder *et al*., 1999).

Arthritis Induced by Systemic Injection of Streptococcol Cell Wall: It is another type of rat model, which includes various form of bacterial cell wall peptidoglycan-polysaccharide induced arthritis in female Lewis rats. The Streptococcal cell wall is one of the most reliable and best characterized experimental models of RA. A single intraperitoneal injection of the Streptococcal cell wall component peptidoglycan-polysaccharide (PGPS), suspended in an aqueous phase will induce a chronic, severe, erosive arthritis in female Lewis rats (Wilder, 1988).

Proteoglycan-induced progressive polyarthritis in rat: As reported by Glant *et al* (1987, 1992), Poole (1989) Mikecz *et al* (1987, 1990), that proteoglycan-induced progressive arthritis and spondylitis in BALB/C mice. This model has few characteristics such as presence of rheumatoid factor and the deposition of immune complexes similar to human rheumatoid arthritis and ankylosing spondylitis as indicated by clinical assessments, immunological parameters, and histopathological studies of diarthrodial joints and spine (Crofford & Wilder, 1997).

Papaya Latex –induced Arthritis: Papaya latex induced inflammation has known to be mediated through prostaglandins. They are also used extensively to evaluate potential of new therapeutic agents for anti-inflammatory activity of the slow-reacting anti-rheumatic drugs (Wilder *et al*., 1999). For this 0.1 ml of 0.25% solution of papaya latex (prepared in 0.05 M sodium acetate buffer, ph 4.5 containing (0.01% thymol) is injected into rat hind paw. The method is sensitive for appraising NSAIDs like aspirin, ibuprofen and steroidal anti-inflammatory drugs, which do not, shows appreciable activity in adjuvant-induced arthritis and other model of inflammation

Complete Freund's Adjuvant (CFA)

CFA is a water-in-oil emulsion, is composed of dried, heat killed mycobacterium tuberculosis, and is used as booster of immune system. CFA is an effective means of potentiating cellular and humoral antibody response to injected immunogens CFA can produce delayed hypersensitivity of extreme severity to a foreign antigen and at times sensitize an animal to its own tissue (Freund, Thompson & Lipton, I955) (auto-immunity). Intradermal injection of CFA causes several painful reactions and leads to tissue damage, skin ulceration, and pulmonary lipid embolism. Thus, the subcutaneous and intraperitoneal routes are always preferred.

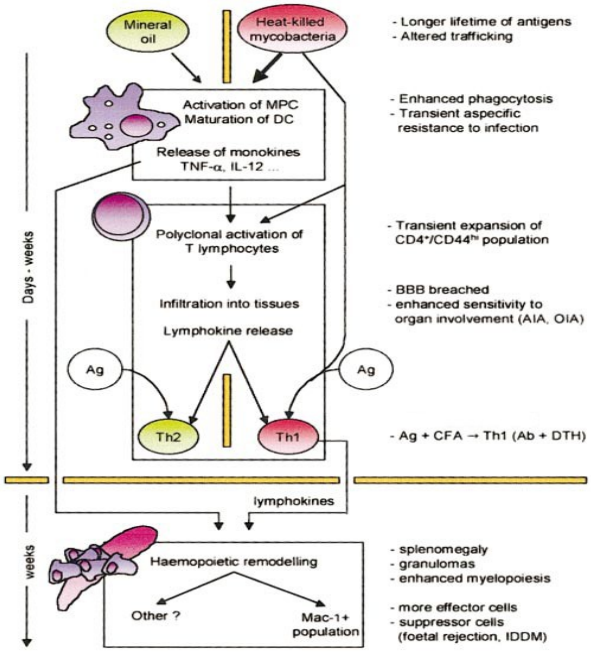


Figure-8: Mechanism of CFA in Arthritis

The adjuvant is known to stimulate production of tumor necrosis factor, which is thought to kill the T-cells responsible for the autoimmune destruction of the pancreatic Beta cells. Adjuvant activity is a result of sustained release of antigen from the oily deposit and stimulation of a local innate immune response resulting in enhanced adaptive immunity. An essential component of this response is an intense inflammatory reaction at the site of antigen deposition resulting from an influx of leukocytes and their interaction with antigen. The use of CFA is an important biologic resource for investigators, which should be used responsibly and with care to avoid or minimize the adverse effects of excessive inflammation.

Statistical Data

More than 30 millions Americans suffer symptoms of one form or another of rheumatic disease. These are inflammatory or degenerative disorders that mainly affect the musculoskeletal system that is such structures as joints, muscles, ligaments, tendon, and bursae. This disease, which afflicts more than 5 million Americans, is characterized by chronic inflammation of the synovial membranes, a layer of loose connective tissue that lines joints and secretes a lubricating fluid. At least 20 million people in India suffer arthritis. The market for pain killer for arthritic pain is approximately Rs.500 crore in India. This means there are an astonishing number people who suffer from musculo-skeletal pain.

Drugs therapy

Conventional medicine treats arthritis with anti inflammatory drugs (most commonly aspirin) and physiotherapy. In severe cases of rheumatoid arthritis, more potent anti-inflammatory drugs are used; nonsteroidal anti- inflammatory drugs such as Indomethacine, cortisone-like drugs, antimalarials gold salts, penicillamine and even experimental cytotoxic drugs. Around the world know of many plants with anti-inflammation and anti-arthriti properties. Unfortunately chemical identification of the anti-inflammatory components are usually given much more attention than thantherapeutic use of the herbs. This is because the plants are viewed as sources of potentially valuable drugs rather than having inherent value in themselves. Some examples of herbal drugs are *Menyanthest trifoliate, Urtice dioica, Harpagophytum procumbens, Chelidonium majus, Commiphora mukkul, Ficus elastica, Lonchocarpus cyanescences, and Scutellaria baicalensis*.

Natural treatments for Arthritis

Though several studies involving drugs have been performed, natural herbs and plants still play a vital role in the treatment of several categories of diseases. The research of natural medicinal sources provides immense positive results and the main reason behind its usage is its property of not showing any side effects. Some of them have been mentioned.

Turmeric: - Turmeric is a wonderful anti-inflammatory that can really ease joints' swelling, which is a major part of arthritis and will help to lessen the pain of arthritis substantially.

***Cayenne pepper*: -** Cayenne pepper has similar positive effects in the treatment of arthritis. These effects have been endorsed by clinical trials.

Ginger: - Ginger contains Zingibain and other phytochemicals, which reduce swelling, which can be very beneficial to arthritis sufferers. Long-term, Ginger is great for protecting the body from free radicals, which do much damage including speeding up the damage of arthritis and causing cancer.

Pineapple: -Pineapple significantly reduces the inflammation of Arthritis flare-ups. However, Pineapple is most effective when combined with other herbal treatments in tablet form as most of its goodness is contained in the stem.

Celery: - Celery, especially the seeds has excellent anti-inflammatory properties that ease stiff joints, which are a main characteristic of arthritis. The seeds work by helping to strip out the solid, which induces a diuretic effect and helps, remove deposits, which collect in the joints of arthritis sufferers. The seeds can be taken in tea or tincture form.

Ginkgo: - Ginkgo increases blood circulation, which helps to ease pain, and swelling in arthritic joints. Comfrey is a great anti-inflammatory boost to the immune system that will stimulate healing in damaged joints.

Few others include -

Arthcare Capsules / Oil: Arthcare is an herbal preparation, which is anti-arthritic and anti-inflammatory and helps in comfortable movement and strengthening of bones, skeletal system, and muscular system by faster penetration into the tissues of the body. It could also be relied upon for, rheumatic and joint pains. It is also a strong antioxidant and increases energy levels. Arthcare capsules are an unique blend of various herbs and are proven safe and effective for those suffering from acute or chronic Rheumatoid Arthritis.

Shallaki (Boswella Serrata): - This herb, also known as Boswella, is an herb found in India and has been used by Ayurvedic physicians over the years for treatment of Rheumatoid arthritis. Extract of Boswella reduced pain, swelling, and morning stiffness when administered to those suffering from Rheumatoid Arthritis. Due to the high anti-inflammatory property of this herb, it inhibits substances causing inflammation, speeds up formation of cartilage, and increases blood supply.

Ashwagandha (Withania Somniferum): - Ashwagandha root is a holistic medicine of Indian origin, belonging to the potato family, which has been used to treat Rheumatoid Arthritis. Because of its high anti-inflammatory property, it is also called “India ginseng”. It rejuvenates and nourishes body tissues and helps in physical fitness.

Tripterygium wilfodii Hook F (TWH); - TWH, a herbal plant growing mainly in South China, was described in ancient Chinese medical texts and has been used widely in China for treatment of joint pain. Patients receiving TWH showed significant improvement in all parameters compared with placebo: tenderness score, swelling count, morning stiffness, and grip strength.

RA-1 (standardized Ayurvedic formulation): - RA-1 is a standardized formulation prepared from purified plant extracts of Withania somnifera (ashwagandha), Boswellia serrata (gugulla), Zingiber officinale (adrak or ginger) and Circuma longa (haldi or circumin).

TAXONOMY AND ETHNO MEDICINE OF

Gaultheria fragrantissima Wall.

SYNONYM:

Gaultheria forrestii Diels

FAMILY:

The species belongs to family "Ericaceae".

SUBFAMILY:

Vaccinioideae

TRIBE:

Gaultherieae

GEOGRAPHICAL DISTRIBUTION:

INDIA **:** This plant is found in the Nilgiris, Travancore (Above 5,000 ft.), North Eastern Himalayas (Khasi Hills).

ASIA Temperate zone **:** China

ASIA Tropical zone **:** From Nepal to Bhutan, 6,000-8,000 ft., Sri Lanka, Myanmar, Vietnam, and Malaysia.

VERNACULAR NAMES:

HIND: Gandapuro

TAM: Kolakkai

KAN : Moolai

SANS: Caramapatra, Nilaphala, Swetapuspa, Talipatra, Hemantaharita

ENG: Indian Wintergreen

JAVA: Gandapuro

NEPAL: Machino

SINHALESE: Welkapuru

DESCRIPTION

It is an evergreen shrub, usually small, low and much branched, bark orange brown, twig pink, young parts glabrous.

LEAVES: Leaves are numerous, persistent, 3.8-6.3 cm., on short stourt petioles, oblong-oval or oblong-lanceolate, rounded at base, obtuse, blunty, apiculate, serrate, glabrous, stiff and coriaceous, venation reticulate, conspicuous, bright green, rather glaucous, and with scattered sunk glands beneath.

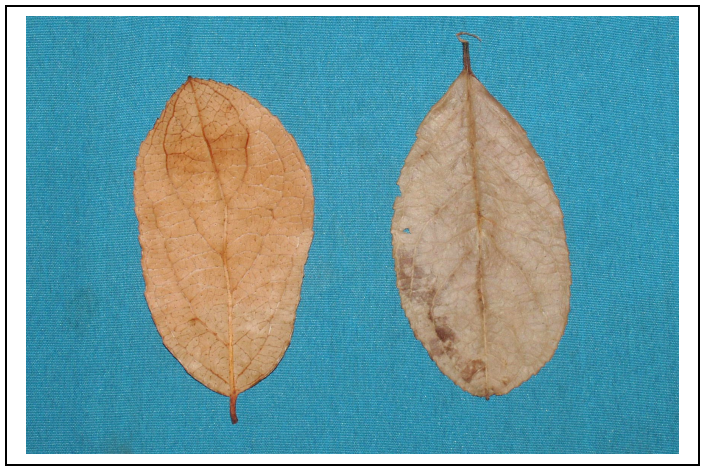


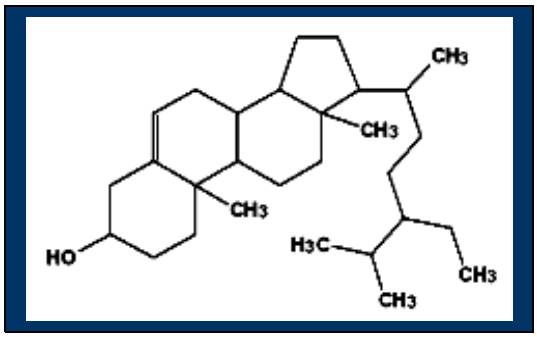
Figure-9: Dried leaves of Gaultheria fragrantissima Wall.

MEDICINAL USES

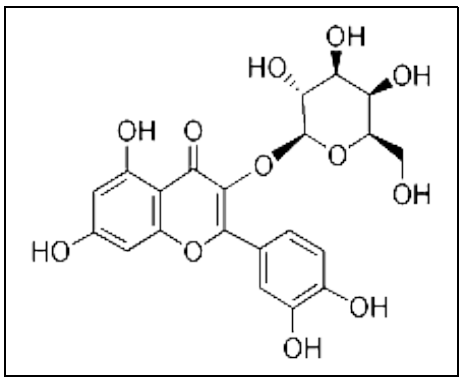
Volatile oil (methyl salicylate) distilled from leaves and shoots traditionally used in acute rheumatism, sciatic and neuralgia. The oil is aromatic, stimulant, and carminative. The oil is also applied as externally in liniments, or in form of suitable ointment. It has powerful antiseptic properties.

Oil of wintergreen and methyl salicylate is moderately potent vermicides against hookworms, which are expelled dead and in a flaccid state; but they are without effect against roundworm.

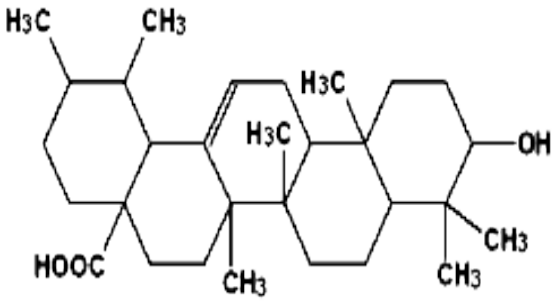
CHEMICAL CONSTITUENTS OF LEAVES



Beta-Sitosterol



Quercetin 3 galactoside



Ursolic acid

LITERATURE REVIEW

Complete Freund's Adjuvant:

- **Geetha et al., (1998).** Effect of triterpenes from Crataeva nurvala stem bark on lipid peroxidation in adjuvant induced arthritis in rats.
- **Ganju et al., (2005).** Anti-inflammatory activity of Seabuckthorn (Hippophae rhamnoides) leaves.
- **Fan et al., (2005).** Effects of an acetone extract of Boswellia carterii Birdw. (Burseraceae) gum resin on adjuvant-induced arthritis in lewis rats.
- **Suk-Jong Suh et al., (2006).** Prevention of collagen-induced arthritis in mice by Cervus Korean TEMMINCK var. mantchuricus Swinhoe.
- **Ramprasath et al., (2006).** Therapeutic effects of Semecarpus anacardium Linn. Nut milk extract on the changes associated with collagen and glycosaminoglycan metabolism in adjuvant arthritic Wistar rats.
- **Ramprasath et al., (2006).** Curative effect of Semecarpus anacardium Linn. Nut milk extract against adjuvant arthritis - with special reference to bone metabolism.
- **Suh et al., (2006).** Effects and mechanisms of Clematis mandshurica Maxim. As a dual inhibitor of proinflammatory cytokines on adjuvant arthritis in rats.
- **Mythilypriya et al., (2007).** Restortative and synergistic efficacy of Kalpaamruthaa, a modified siddha preparation, on an altered antioxidant status in adjuvant induced arthritic rat model.
- **Rasool & Varalakshmi, (2007).** Protective effect of Withania somnifera root powder in relation to lipid peroxidation, antioxidant status, glycoproteins and bone collagen on adjuvant induced arthritis in rats.
- **Kim et al., (2007).** Effect of Cervus korean TEMMINCK var. mantchuricus Swinhoe on protease activities, antioxidant and free radical damages in rheumatis arthritis rats.
- **Rajkapoor et al., (2007).** Effect of Bauhinia variegate on complete freunds adjuvant induced arthritis in rats.

- **Narendhirakannan et al., (2007).** Anti-inflammatory and lysosomal stability actions of Cleome gynandra L. studied in adjuvant induced arthritic rats.

- **Manal F et al., (2008).** Study of the immunomodulatory and anti-inflammatory effects of evening primrose oil in adjuvant arthritis.

- **Mythilypriya et al., (2008).** Efficacy of Siddha formulation Kalpaamruthaa in ameliorating joint destruction in rheumatoid arthritis in rats.

- **Mythilypriya et al., (2008).**Salubrious effect of Kalpaamruthaa, a modified indigenous preparation in adjuvant-induced arthritis in rats—A biochemical approach.

***Gaultheria fragrantissima* Wall.**

- **Samba Murthy** & **Rajendra Babu**, (**1970**, **1972);** Chemical investigation of the leaves of *G. fragrantissima* Wall Leaves yield β-sitosterol, quercetin-3-galactoside and ursolic acid. Fresh leaves were repeatedly extracted with alcohol. The evaporated extract was defatted with petroleum ether. The unsaponifiable matter obtained from the petroleum ether soluble, yield β-sitosterol. The petroleum ether insoluble residue of the original extract was treated with hot water. The aqueous extract was shaken with ethyl acetate, which gave quercetin-3-galactoside. The water insoluble fraction was taken up in a water-immiscible organic solvent mixture and the solution was shaken with alkali when a precipitate separated at the interphase. Decomposition of the interphase precipitate yields ursolic acid.

- **Bhakuni et al**., **(1971);** Screening of Indian plants for biological activity: part III. Ethanol extract (50 %) of plant part excluding root, of *G. fragrantissima* Wall. have been tested for wide variety of biological activity including anticancer (human epidermoid carcinoma of nasopharynx in tissue culture, walker carcinoma 256 in rat, L-1210 lymphoid leukemia in mice), chemotherapeutic and pharmacological activities (hypoglycemic, diuretic, supramaximul electroshock seizure pattern test, hypothermia, analgesia).

- **Baruah**, (**1976).** Fresh leaves, essential oil, 0.7-0.8 %; containing methyl salicylate, 99.6 %.

- **Polunin** & **Stainton**, (**1984)**. The leaves of *G. fragrantissima* Wall. are medicinally important due to the presence of an active principle compound, methyl salicylate and are used in the preparation of pain balms.

- **Wollenweber** & **Kohorst**, (**1984)**. From the fresh leaves of *G. fragrantissima* Wall. Two new flavonoids have been identified.

- **Ichiyama et al**., (**2002**). Effects of topical analgesics on the pressor response evoked by muscle afferents. Pressor responses are reflexly evoked by the activation of groups III and IV muscle afferents, which are also known to mediate nociceptive responses. In this experiment, the effects of analgesic balm (AB) application on these responses were investigated without the interference of other types of anesthesia or effects from the higher brain.

- **Battino et al**., (**2002)**. *In vitro* antioxidant activities of mouth rinses and their components. Several forms of periodontal diseases (PD) are often associated with activated phagocytosing leukocytes and contemporary free radical production. The aim of the present study was to determine possible antioxidant activity (AA) of a number of antiseptic mouth rinses and of their stated active principles (AP), regardless of their efficacy as antimicrobial agents.

- **Bhatt et al**., (**2004**). An evaluation of characteristics of indigenous shrub of the North Eastern Himalayan region was carried out to identify potential for firewood production. A fuelwood value index (FVI) was defined as the Calorific value X Desity / Ash content.

- **Sharma** & **Kolhapure**, (**2005)**. Evaluation of the efficacy and safty of Rumalaya gel in the management of acute and chronic inflammatory mucoskeletal disorder: an open, prospective, noncomparative, phase III clinical trial.

- **Apte et al**., (**2006**). Study of chloroplast and mitochondrial DNA diversity and bioprospecting potential in the natural population of *G. fragrantissima* Wall. in the two hot spot in India.

- **Prabuseenivasan et al**., (**2006**). In vitro antibacterial activity of essential oil of *G. fragrantissima* Wall. Were screened against four gram-negative bacteria

(*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) and two gram-positive bacteria *Bacillus subtillis* and *Staphylococcus aureus* at four different concentrations (1:1, 1:5, 1:10 and 1:20) using disc diffusion method.

- **Rana** & **Blazque**, (**2006)**. Chemical composition of the essential oil of *G. fragrantissima* Wall. leaves.

- **Paulsamy et al**., (**2007**). Genetic variation and degree of correlation in four ecological variants (ovate, lanceolate, elliptic-lanceolate and oblanceolate leaf types) of *G. fragrantissima* Wall. In Nilgiri Biosphere Reserve, Western Ghats, India.

AIM AND SCOPE OF STUDY

Rheumatoid arthritis (RA) is a chronic autoimmune disorder with swelling, pain, stiffness, deformity, and cartilage destruction. Rheumatoid arthritis affects 1% of the worldwide general population and it is 2 to 3 times more common in women than in men. However, the cause of RA, still remains unknown, it is accepted that reactive oxygen species has been proved to play the major role in pathogenesis of RA. Lysosomes are membrane bound organelles that contain digestive enzymes, which are stored in the sub cellular organelles termed lysosomal granules. It has been proved that Lysosomal enzymes play an important role in adjuvant induced arthritis by altering the metabolism of glycoprotein and glyaminoglycans. It has been proved that CFA induced arthritis significantly improve the level of inflammation and increases the extracellular activities of lysosomal enzymes. This eventually leads to membrane damage, which finally leads to cartilage destruction and bone resorption.

Complete Freunds Adjuvant (CFA) is composed of dried, heat killed mycobacterium tuberculosis is used as booster of immune system. Freund's complete adjuvant (FCA) can produce delayed hypersensitivity of extreme severity to a foreign antigen and at times sensitize an animal to its own tissue. Several instances have been reported where intradermal injection of CFA causes several painful reactions and leads to tissue damage, skin ulceration, and pulmonary lipid embolism. This eventually leads to rheumatoid arthritis and thus it is used as a model of chronic inflammation in rats for the pharmacological and pathophysiological control of inflammatory responses. Strong relation between the efficiency of therapeutic agents in this model and rheumatoid arthritis in humans quotes for its worldwide utilization. Several evidences suggested that arthritis induced by CFA leads to significant increase in the level of pro-inflammatory cytokines in the synovial fluid. These are mediated by primarily by activation of “effectors cells” macrophages and connective tissue (fibroblast). Previous reports proved that herbal therapies provide an approach in the treatment of various ailments including arthritis.

Gaultheria fragrantissima Wall (GF) (family-Ericaceae) commonly known as Indian wintergreen oil, is cultivated on high altitude. It is an evergreen shrub, cultivated for medicinal uses against acute rheumatism, sciatic and intercostals neuralgia. The

leaves of plant *Gaultheria fragrantissima Wall*, has been considered most active for therapeutic purpose. Several reports suggest that the plant *Gaultheria fragrantissima Wall* possess antidiabetic, anticancer, diuretic, hypothermic, analgesic and antiepileptic activities. The major constituents present are beta-sitosterol, quercetin-3-galactoside and ursolic acid. Reports suggest that beta-sitosterol posse's antioxidant and anti-inflammatory activity in oedema. It also shows potential activity against rheumatoid arthritis in rats. Moreover, it has been well documented that quercetin-3-galactoside (polyphenolic flavonoid), and ursolic acid (pentacyclic triterpenoid) scavamges free radicals. Cumulative studies prove that ursolic acid is effective in rheumatoid arthritis as hepatoprotective and cardioprotective activities. In our previous published data suggest the antioxidant potential of *Gaultheria fragrantissima Wall* against CFA induced arthritis in rat liver. In current study the Efficacy of *Gaultheria fragrantissima* Wall. on lysosomal destabilization and inflammation by CFA induced arthritis in rat liver.

PLAN OF WORK

1. **Collection, identification and authentication of *Gaultheria fragrantissima* Wall. Leaf**

2. **Extraction of dried plant material**

    - Hot continuous percolation using Soxhlet apparatus by ethanol (70% v/v) as solvent.

3. **Estimation of Lysosomal Enzymes**

    - β-D-glucuronidase
    - Acid phosphatase
    - β-D-N-acetyl glucosaminidase
    - Cathepsin D

4. **Estimation of Cytokine Levels**

    - Estimation of TNF-α
    - Estimation of IL-6

5. **Estimation of Glycoprotein**

    - Hexose
    - Hexosamine
    - Sialic acid

6. **Histopathological studies**

7. **X- ray study**

MATERIALS AND METHODS

COLLECTION AND AUTHENTIFICATION OF PLANT MATERIAL

Gaultheria fragrantissima Wall leaf was collected from Survey of Medicinal Plants & Collection Unit, Ooty, Tamilnadu, India and authenticated (voucher no of the specimen: PARC/2008/129) by Plant Anatomy Research Centre (PARC) Medicinal plant Research Unit, Chennai, India.

Extraction procedure:

The collected sample of *Gaultheria fragrantissima* Wall leaf was shade dried for 1-2 weeks and grind to make a coarse powdered form. The coarsely powder was subjected to hot continuous percolation using ethanol (70% v/v) as solvent. The extract was concentrated under vacuum, and then dried in a vacuum desiccators (yield 9.3% w/ w), and stored in an air- tight containers for further use.

BIOCHEMICAL ESTIMATIONS

Animals:

The study was performed on male albino rats of Wistar strain (average weight of 150-180 g), obtained from Experimental Animal Care Centre, Vel's College of Pharmacy, Chennai, India. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Culture, Chennai. The animals were housed in standard laboratory conditions 12 ± 1 h day and night rhythm during the experimental period and they were given food and water supplied by Hindustan Lever Ltd., Mumbai, India under the trade name Gold Mohur rat feed and water *ad libitum*.

Experimental Protocol:

Animals were divided into four groups of six animals in each group as follows:

Group I: Control rats received water and food.

Group II: Arthritic rats served as induced [arthritic syndrome was induced by single subcutaneous injection of 0.1 mL of CFA (10 mg of heat killed *Mycobacterium*

tuberculosis mL^{-1} of paraffin oil) into the plantar surface of right hind paw, exactly on the first day of the experimental period].

Group III: Control rats administered with extract of GF (200 mg kg-1 $days^{-1}$) for 28 days.

Group IV: Arthritic rats treated with GF (200 mg $kg^{-1}day^{-1}$) orally for 14 days (from day 14 to day 28).

After the experimental period all, the animals were anesthetized and decapitated. Liver tissues were immediately excised and rinsed in ice-cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCL buffer, (pH 7.4) and aliquots of this homogenate were used for the assay. Blood was collected and serum was separated for analysis of biochemical parameters.

Biochemical Investigation procedure

Isolation of lysosome

Lysosomal fraction was isolated by the method of Wattiaux (1977).

Reagents

1.	Sucrose solution	:	0.25 M
2.	Potassium chloride	:	1.15%

Procedure

Fresh liver tissue was homogenised in 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3,000 × g for 10 minutes in a refrigerated Beckman J2-21 centrifuge. The pellet was removed and re-homogenised and resuspended as before. The supernatants were combined and centrifuged again at 15,000 × g for 20 minutes. The lysosomal pellet obtained was suspended in 1.15% KCl, homogenised and used for the estimation of enzymes.

Assay of β-D-glucuronidase

The activity of β-D-glucuronidase was determined by the method of Kawai and Anno (1971).

Reagents

1. 0.5 M NaOH
2. Sodium acetate buffer : 0.1 M, pH 5.0
3. *p*-nitrophenyl β-D-glucuronide : 2 mM
4. Glycine - NaOH buffer : 0.2 M, pH 11.7 containing 2 M SDS

Procedure

Known aliquot (0.2 ml) of the enzyme source was added to 0.5 ml of incubation buffer containing 2 mM substrate (final concentration) and incubated at 37°C for 2 hours. The substrate p-nitrophenyl β-D-glucuronide was dissolved in 0.1M acetate buffer. At the end of the incubation period, the reaction was stopped by the addition of 4.0 ml of 0.2 M glycine-NaOH buffer and the contents were centrifuged. To the aliquots of supernatants, 0.5 M NaOH was added and the absorbance was measured at 410 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as μmoles of p-nitrophenol liberated/mg protein.

Assay of Acid phosphatase (ACP)

Acid phosphatase was assayed by the method of King (1965a).

Reagents

1. Acetate buffer : 0.1 M, pH 4.8
2. DPP : 0.01 M
3. Folin's phenol reagent : Commercial reagent, 1:2 dilution
4. Sodium carbonate : 15% solution

5.	Standard phenol	:	100 mg of recrystallised phenol in 100 ml of water.
6.	Working standard	:	100 µg of phenol/ml was prepared by proper dilution.

Procedure

The incubation mixture of final volume 3.0 ml containing 1.5 ml of buffer, 1.0 ml of substrate and required amount of the enzyme source were incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent. To the control tubes, enzyme was added after arresting the reaction. The contents were centrifuged and 1.0 ml of 15% sodium carbonate was added to the supernatant. The mixture was incubated for 15 minutes at 37°C and the colour developed was read at 640 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as IU/l for serum and µmoles of phenol liberated/h/mg protein for tissue.

Assay of β-D-N-acetyl glucosaminidase

The activity of β-D-N-acetyl glucosaminidase was assayed by the procedure of Moore and Morris (1982).

Reagents

1.	Glycine - NaOH buffer	:	0.2 M, pH 11.7 containing 2 M SDS
2.	Citrate buffer	:	0.1 M, pH 4.5
3.	Standard	:	6 mM p-nitrophenol
4.	Substrate	:	2 mM p-nitrophenyl β-D-N-acetyl glucosaminide

Procedure

Known aliquots (0.2 ml) of the enzyme source was added to 0.5 ml of incubation buffer containing 2 mM substrate (final concentration) and incubated at 37°C for 2 hours. At the end of the incubation period, the reaction was stopped by the addition of 4.0 ml of glycine-NaOH buffer and the contents were centrifuged. To the aliquots of supernatants, 0.5 M NaOH was added and the absorbance was measured at 410 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as µmoles of p-nitrophenol liberated/h/ mg protein.

Assay of Cathepsin D

Cathepsin D activity was determined by the method of Sapolsky *et al.* (1973).

Reagents

1.	NaOH	:	1 N
2.	TCA	:	10%
3.	Substrate buffer, pH 3.0	:	1.5% haemoglobin in 0.1 M acetate
4.	Folin's phenol reagent	:	Commercial reagent, 1:2 dilution

Procedure

Known aliquots (0.2 ml) of the enzyme source was incubated with 1.5% haemoglobin for 2 hours. The enzyme activity was arrested by the addition of 10% TCA and the liberated TCA soluble products were filtered and neutralized with 1 N NaOH. The tyrosine content of the filtrate was determined using Folin's phenol reagent essentially employing the procedure of Lowry *et al.* (1951) as described. The blue colour developed was read at 660 nm in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as µmoles of tyrosine liberated/ h/100 mg protein.

Assay Of Tumour Necrosis Factor - A (TNF - α)

The level of TNF-α was measured by using enzyme linked immunosorbent assay as described earlier in section using mouse monoclonal antibody against rat TNF-α (1:5000 dilution) and Goat-ant mouse IgG-HRP conjugate.

The level of TNF-α was expressed as ng/mg protein using a calibration curve obtained from standard TNF-α.

Assay of Interleukin – 6; (IL-6)

The level of IL-6 was measured by using enzyme linked immunosorbent assay as described earlier using mouse monoclonal antibody against rat IL-6 (1:5000 dilution) and Goat-antimouse IgG-HRP conjugate.

The level of IL-6 was expressed as ng/mg protein using a calibration curve obtained from standard IL-6.

ANALYSIS OF GLYCOPROTEIN COMPONENTS

Estimation of Hexose

Hexose was estimated by the method of Neibes (1972).

Reagents

1. Orcinol-sulphuric acid reagent :

   Reagent A : Sulphuric acid-water mixture (3:2 v/v)

   Reagent B : 1.6 g of orcinol in 100 ml of water

   Reagents A and B were mixed in the ratio of 15:2 (v/v)

2. Hydrochloric acid : 4 N

3. Sodium hydroxide : 4 N

4. Standard hexose : Equal quantities (10 mg/100 ml) of galactose and mannose were dissolved in water to give a concentration of 100 µg/ml.

Procedure

0.5 ml of the neutralized sample was made up to 1 ml with water and 2 ml of orcinol-sulphuric acid reagent was added to the tubes in ice-cold condition very slowly. The tubes were read at 540 nm after 20 minutes in a Shimadzu UV spectrophotometer. Standard hexose in the range of 20-100 µg/ml and blank were treated in the same manner.

The values were expressed as mg/100mg of dry defatted tissue.

Esitmation of Hexosamine

Hexosamine was estimated by the method of Wagner (1979).

Reagents

1. Acetyl acetone reagent: 3.5% acetyl acetone in 1 N trisodium phosphate and 0.5 N potassium tetraborate were mixed in the ratio of 98:2 (v/v).
2. Ehrlich's reagent: 320 mg of p-dimethyl aminobenzaldehyde was dissolved in 21 ml of isopropanol and 3 ml of concentrated hydrochloric acid.
1. Standard hexosamine: Galactosamine hydrochloride solution containing 10 mg/100 ml was prepared.
2. Hydrochloric acid : 4 N
5. Sodium hydroxide : 4 N

Procedure

To 0.8 ml of the neutralized sample, 0.6 ml of acetyl acetone reagent was added. Blank contained 0.8 ml of distilled water. The tubes were heated in a boiling water bath for 30 minutes. The tubes were cooled and then 2 ml of Ehrlich's reagent was added. The colour developed was read at540 nm in a Shimadzu UV spectrophotometer. Standard galactosamine in the range of 10-40 µg, were also processed in the same manner.

The values were expressed as mg/g of dry defatted tissue.

Estimation of Sialic acid

Sialic acid was estimated by the method of Warren (1959).

Reagents

1.	Sodium meta periodate	:	0.2 M in 9 M phosphoric acid
2.	Sodium arsenite	:	10% in 0.5 M sodium sulphate in 0.1 N sulphuric acid
3.	Thiobarbituric acid	:	0.6% in 0.5 M sodium sulphate
4.	Acidified butanol	:	100 ml of butanol containing 5 ml of concentrated HCl
5.	Standard	:	N-acetyl neuraminic acid solution containing 100 µg/ml in distilled water.

Procedure

To 0.2 ml of the hydrolysed sample, 0.2 ml Sodium meta periodate reagent was added and kept at 37°C for 20 minutes. Then 0.2 ml of sodium arsenite was added and shaken well. After adding 3 ml of thiobarbituric acid, the tubes were heated in a boiling water bath for 15 minutes. The tubes were cooled and 5 ml of acidified butanol was added. The absorbance of the pink butanolic phase after centrifugation was read at 540 nm in a Shimadzu UV spectrophotometer. Standards and blank were also processed in the same manner.

The values were expressed as mg/g of dry defatted tissue.

HISTOPATHOLOGICAL STUDIES

The ipsilateral (injected) proximal interphalangeal joints were excised, fixed in 10% neutral buffered formalin, decalcified in 5% nitric acid and processed paraffin wax embedding. Sections were cut at 3 μm thickness, flattened and adhered to the slides. They were stained with hematoxylin-eosin and viewed under light microscope for histopathological changes.

X-RAY STUDIES

Before sacrificing the animals, X-ray were taken at the joints of hind paw of animals for evaluating the bone damage.

STATISTICAL ANALYSIS

All the grouped data were statistically evaluated with Statistical Package for Social Sciences (SPSS), Version 7.5. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. A 'P' value of less than 0.05 was considered to indicate statistical significance. All the results were expressed as mean + S.D. for six animals in each group.

RESULTS

Table-1 represents Effect of CFA and GF on body and tibia weight in control and experimental animals, in CFA induced arthritis (Group-II) the weight are significantly reduced ($p < 0.05$) in comparison to control (Group I). Administration of GF extract (Group IV) increased the weight significantly ($p < 0.05$) near to normal level as control (Group I). GF extract (Group III) treated alone shows normal as control (Group I).

Table-2 represents the effect of CFA and GF on Lysosomal enzymes in liver of control and experimental animals. The levels of Lysosomal enzymes such as N-acetylglucosaminidase (NAG), betaglucuronidase, acidphosphatase, and cathepsin-D were significantly ($p < 0.05$) increased in Group-II in comparison to Group-I in liver. After treatment with GF, these lysosomal enzyme levels were reduced near to normalcy comparing with Group-II. GF extract treated alone (Group III) shows normal as control (Group I).

Table-3 represents the Effect of CFA and GF on glycoproteins in liver of control and experimental animals. The marked increase in levels of glycoproteins such as Hexose, Hexosamine, and Sialic acid, significantly ($p < 0.05$) in Group-II (CFA induced) rat was compared to Group-I, while Group-IV (GF extract) shows reduced levels of these enzymes compared to Group-II. GF extract treated alone (Group III) shows normal as control (Group I).

Figures-10 and figure-11 represents the effect of CFA and GF on TNF-α and IL-6 level in serum of control and experimental animals. The levels of pro-inflammatory cytokines such as TNF-α and IL-6 are significantly ($p < 0.05$) increased in Group-II when compared to Group-I and it is significantly ($p < 0.05$) restored in Group-IV compared to Group-II. GF extract treated alone (Group III) shows normal as control (Group I).

Plate I shows Histopathological examinations of proximal interphalangeal joints in control and experimental animals. In Figure-12 (control) showing normal articular surface with joint space compared to Figure-13 (CFA induced) where joint showing damaged articular cartilage and space with adjacent area of inflammatory granulation

tissue. Figure-14 (GF extract 200mg/kg/bw) showing that damage is restrained but the inflammatory granulation tissue is present less than Figure-13 (CFA induced).

Plate II shows X- ray study of rat foot region in control and experimental groups. The pictures shows marginal erosion in the joint and swelling in CFA induced arthritic rats (Group II) in comparison with control (Group I). In GF treated arthritic rats, (Group III) these changes were normalized which indicates its protective effect. GF extract treated alone (Group III) shows normal as control (Group I).

DISCUSSION

Rheumatoid arthritis has been proved as an inflammatory responses mediated autoimmune disease,(Levinson et al., 1994), characterized by leukocyte infiltration, swelling, pain, stiffness, destructive damage of joint tissues, as well as hyper proliferation of the synovial cells (Page c et al 2002, Koch AE et al 1998) and the initiation of a chronic inflammatory state that involves overproduction of pro-inflammatory cytokines (R, Fleischmann et al 2004,J.M Dayer et al 2003). Mounting evidences proved that CFA induced RA may generate enormous free radicals and decrease in antioxidant scavenger leads to oxidative stress (Bandyopadhyay U, et al, 1999). Several evidences proved that free radicals and their derivatives play an important role in pathogenesis of the autoimmune disorders (Blake DR, et al 1981). The hydroxyl radicals formed by the Fenton reaction of ferrous ions and H_2O_2, leads to iron deposition produce deterous effect to proteins, DNA, and the membrane lipids. These radicals are also involved in autoantibody production, which inactivates α-1 antiproteinases, conformations, antigenicity, and destruction of synovial antioxidants (Gutteridge J.M.C.et al, 1986, G.Tsuji et al, 2006). It has been report that antioxidants play an important role in free radical scavenging activity and our previous studied proved that Gaultheria Fragrantissima Wall (Ericaceae) possesses antioxidant activity against CFA induced arthritis in rats (Shanmugarajan et al 2008). However, the current study demonstrated the anti-inflammatory and lysosomal membrane stabilizing effect of GF against CFA induced arthritis.

Oxygen free radicals (OFRs) are implicated as mediators of hepatic injury in rheumatoid arthritis shown by our previous study (Rainsford et al, 1982). Reactive oxygen species react with membrane lipids causing lipid peroxidation (Heliovaara et al, 1994)and subsequent leads to membrane destruction (Marnett et al,1999). Lysosomes are membrane bound structures that contain several hydrolytic enzymes that are involved in the digestion of endogenous and exogenous compounds (R.T.Narendhirakannan et al, 2006,). Cumulative studies reported that these enzymes are important mediators of hepatic injury and its release into cytoplasm stimulate the generation of stress-mediated radicals. These enzymes degrade the material ingested in the phagocytic vacuole which is due to production of immune complexes. This complex leads to release of certain factors that leads to membrane damage by attracting

leukocytes thereby releasing the lysosomal enzymes (T.Guardia et al, 2001). Elevated lysosomal enzymes in the extracellular fluid occur because of decreased lysosomal membrane stability (Surinder Kumar yogeeta et al, 2006,). Lysosomal enzymes like betaglucuronidase; N-acetylglucosaminidase; acidphosphatase, and cathepsin-D were found to be closely related with RA (A.F.Safina et al 1992). Betaglucuronidase in the serum acts as an immediate indicator to check the extent of effectiveness of drugs in RA conditions (A Falkenbach et al, 86, (1991).The activities of serum acid hydrolases is increased in CFA induce arthritis rats (Yasuda et al, 2000). The finding shows increased levels of N-acetylglucosaminidase (NAG), betaglucuronidase, acidphosphatase, and cathepsin-D in CFA induced arthritis rats may be due to the lipid deterioration of lysosomal membrane. Several studies reported that flavonoid and β-sitosterol play an important role in stabilization of lysosomal Membrane (Jagetia et al, 2002,). It has been well proved that ursolic acid posses membrane stabilization activity (Han et al 1997, Balaneehru and Nagarajan et al 1992,). The pre treatment of GFA shows the reduced levels of lysosomal enzymes by the presence of these phytoconstituents may be responsible for decrease the level of lysosomal enzyme and helps in maintains of structural integrity of cell membrane in CFA induce arthritis.

Cumulative studies showed that in arthritic conditions neutrophils impart in the production of Reactive Oxygen species by amplifying the activities of lymphocytes and macrophages (Nurcombe et al, 1991). Neutrophils also increase the levels of cytokines (TNF-α, IL-6) by proliferation of synoviocytes (O'Reilly MS, 1997, et al), which ultimately generates H_2O_2 by fibroblasts and Chondriocytes (Tiku et al, 1990,). Ample literature prostrated that oxygen derived free radicals modulate the level of cytokines (TNF-α) (Meier B, et al 1989,).Reports imply that the pathogenesis of RA is brought about by the activity of a complicated network of cytokines (Choy and Panayi, 2001). TNF- α and IL-6 are acknowledged to be the primary cytokines responsible for mediating the marked destruction of cartilage and bone in arthritis (Carteron, 2000). Literature data suggest that CFA induced RA has been shown an enhanced levels of TNF-α in plasma which leads to simultaneous increase in superoxide ions thereby increasing the intensity of oxidative stress (Miesel et al, 1996 a,b). Enhanced levels of TNF- α stimulate the generation of other cytokines, induce endothelial adhesion molecules, stimulate collagenase and excite osteoclast differentiation (Bazzoni and Beutler, 1996 ;). This leads to activation of the collagenase enzymes and disturbs the

integrity of collagen, which grounds for decrease in the collagen levels and therefore leads to cartilage destruction (Schuna A, A, et al 2000; Geetha T, et al 1999) as well as it helps in enhancing the activity of bone resorption (Ralston & Grabowski, 1996). Hence, the obstruction of TNF- α has an added effect on inflammation than the blockade of other cytokines.

Substantial evidence stated that increased levels of cytokines implies the generation of nitric oxide radicals by activation of inducible nitric oxide synthase (Rediske et al, 1994, Sakurai et al, 1995, Perkins et al, 1998, Stichtenoth Frolich, 1998). The generated nitric oxide radical react with super oxide ions leads to the formation of peroxynitrates which is a potent oxidant leads to significant nitration of tyrosine residue, protein and lipid destruction indicate lipid peroxidation (Beckman et al 1994, Pryor WA et al 1995, Ischiropoulos H et al 1992;). It has been well documented that arthritis induced by CFA leads to production of cytokines such as TNF-α and IL-6 which may be activation of transcription factors or generation of oxygen-derived free radicals (Morel Baroiki, 1998, Chen et al, 1999). This was observed in current study on CFA induced arthritis shows the accelerated levels of TNF-α and IL-6. Several studies report that both β-sitosterol and Ursolic acid act as antioxidants, which have potent anti-inflammatory activities and help to reduce the levels of pro-inflammatory cytokines (Gupta MB et al 1996, Bouic PJD et al 1999, Chattopadhyay D et al, 2005, Arunachalam G et al, 2002; Chattopadhyay D et al 2002). In current study deleterious effect was conquered over CFA induced RA after the treatment with GF, which is due to its anti-inflammatory activity of phyto-constituents such as β-sitosterol, and ursolic acid, which is already reported.

Glycoproteins are protein carbohydrate-linked active macromolecules present in the cells in both soluble and membrane-bound forms. Aggumented reports stated that it plays an important role in secretion and absorption of macromolecules, cell differentiation and signal transduction among cells (Mittal et al., 1996**).** Substational reports suggest that glycoprotein and glycoaminoglycans are responsible in maintaining the structural firmness of collagen fibrils (D.S.Jackson et al 1968,). The increase in the levels of glycoproteins (hexose, hexosamine, and sialic acid) occurs in case of adjuvant induced arthritis as well as in the case of inflammation which may be attributed to

connective-tissue-activating factor is observed in CFA induce rats in agreement with previous reports (Caster et al., 1958). Treatment with GF extract however proved to be beneficial in lowering the enhanced levels of glycoproteins to normalcy which may be due to the presence of several phyto-constituents.

SUMMARY AND CONCLUSION

To summarize, these finding of this current exploration suggest the excessive free radical imposed stress by CFA induce arthritis resulted in bone erosion, lysosomal damage and increase serum pro-inflammatory cytokines levels In contrast, GF treatment regain the lysosomal structural integrity and anti-inflammatory effect. This sumpshot shows the light on the fact that GF might be beneficial in the stress condition implied by CFA induce arthritis.

Concluding our study GF treatment regains the liver lysosomal structural integrity and anti-inflammatory effect induced by CFA induced arthritis in rat.

LIST OF TABLES

LIST OF FIGURES

PLATE-I: HISTOPATHOLOGY REPORTS

PLATE-2: X- RAY REPORTS

TABLES AND FIGURES

Table-1: Effect of CFA and GF on body and tibia weight in control and experimental animals

Groups	Group I (Control)	Group II (CFA Induced)	Group III (GF extract alone)	Group IV (CFA+GF 200mg/ kg)
Find body weight (gm)	141.32±7.83	111.11±4.86^{*a}	141.64±6.25^{NS}	132.70±6.08^{*b}
Whole Tibia weight (mg)	256.23±9.14	200.82±6.17^{*a}	255.83±8.65^{NS}	237.65±7.34^{*b}

Values are expressed as mean ± S.D. for six animals.

a= Comparison are made between Group-I and Group-II;

b= Comparison are made between Group-II with Group-IV.

*** represent the statistical significance at $p < 0.05$**

NS represents non-significance at $p < 0.05$.

Table-2: Effect of CFA and GF on Lysosomal enzymes in liver of control and experimental animals

Groups	β-D-glucuronidase	Acid phosphatase	β-D-N-acetyl glucosaminidase	Cathepsin-D
Group-I (Control)	8.52±0.26	92.20±3.18	22.49±1.27	24.45±1.44
Group-II (CFA Induced)	20.21±0.01^{*a}	174.51±4.70^{*a}	40.44±0.03^{*a}	49.31±2.96^{*a}
Group-III (GF extract alone)	8.53±0.22^{NS}	91.73±4.06^{NS}	23.33±1.03^{NS}	24.20±1.71^{NS}
Group-IV (CFA+GF, 200mg/ kg)	11.61±0.89^{*b}	110.30±2.79^{*b}	29.76±1.67^{*b}	30.44±2.28^{*b}

Values are expressed as mean ± S.D. for six animals. Units: β-D-glucuronidase (µmoles of tyrosine liberated/ h/100 mg protein); Acid phosphatase (IU/l for serum and µmoles of phenol liberated); β-D-N-acetyl glucosaminidase (µmoles of p-nitrophenol liberated/h/mg protein); Cathepsin-D (µmoles of tyrosine liberated/ h/100 mg protein).

a=Comparison are made between Group-I and Group-II;

b= Comparison are made between Group-II with Group-IV.

*** represent the statistical significance at $p < 0.05$**

NS represents non-significance at $p < 0.05$.

Table-3: Effect of CFA and GF on Glycoproteins in liver of control and experimental animals

Groups	Hexose	Hexosamine	Sailic acid
Group-I (Control)	145.31±7.13	0.22±0.006	38.09±4.56
Group-II (CFA Induced)	181.87±9.81^{*a}	0.42±0.014^{*a}	59.43±3.71^{*a}
Group-III (GF extract alone)	145.18±5.08^{NS}	0.22±0.014^{NS}	39.62±3.73^{NS}
Group-IV (CFA+GF 200mg/kg)	150.40±4.85^{*b}	0.28±0.010^{*b}	41.98±3.63^{*b}

Values are expressed as mean ± S.D. for six animals. Units: Hexose **(Units mg/100mg protein);** Hexoseamine **(Units mg/g protein);** Sailic acid **(Units mg/g protein).**

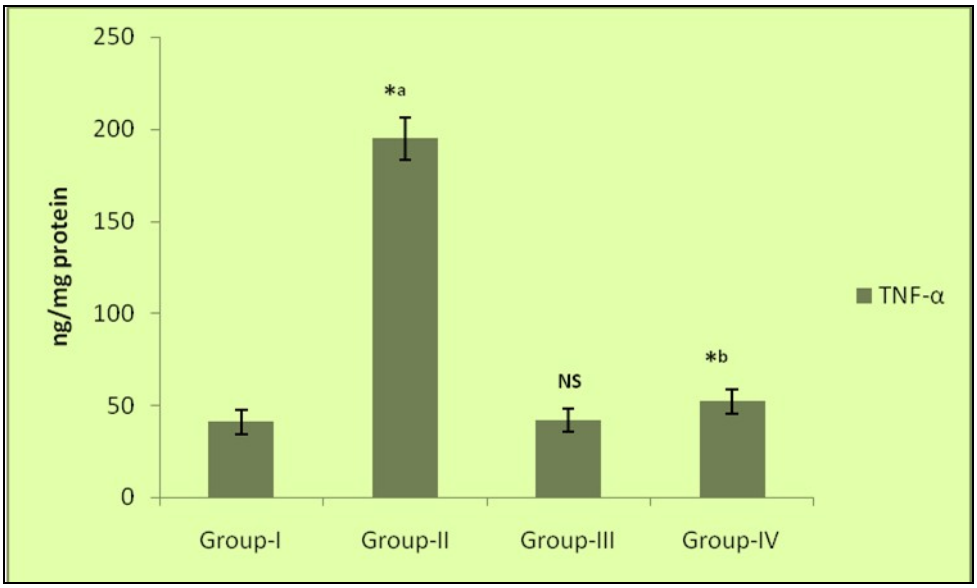
a=Comparison are made between Group-I and Group-II;

b= Comparison are made between Group-II with Group-IV.

*** represent the statistical significance at $p < 0.05$**

NS represents non significance at $p < 0.05$.

FIGURE-10: Effect of CFA and GF on TNF-α level in serum of control and experimental animals



Values are expressed as mean ± S.D. for six animals. Units: TNF-α (ng/mg protein);

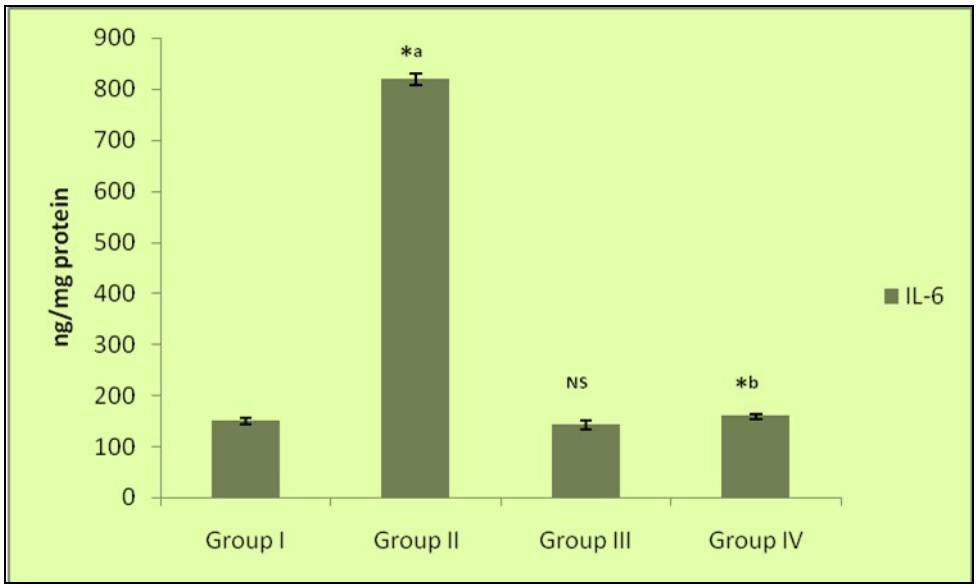
a=Comparison are made between Group-I and Group-II.

b= Comparison are made between Group-II with Group-IV.

*** represent the statistical significance at $p < 0.05$.**

NS- represents non-significance at $p < 0.05$.

FIGURE-11: Effect of CFA and GF on IL-6 level in serum of control and experimental animals



Values are expressed as mean ± S.D. for six animals. Units: IL-6 (ng/mg protein);

a = Comparison are made between Group-I and Group-II.

b = Comparison are made between Group-II with Group-IV.

*** represent the statistical significance at $p < 0.05$.**

NS represents non-significance at $p < 0.05$.

PLATE-1: HISTOPATHOLOGICAL EXAMINATION OF INTERPHALANGEAL JOINTS IN CONTROL AND EXPERIMENTAL ANIMALS

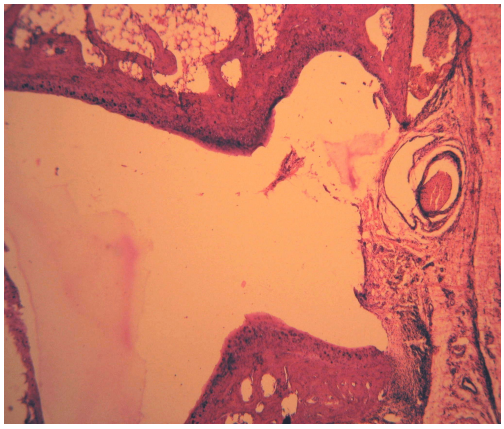


Figure-12: (Group-I) Control

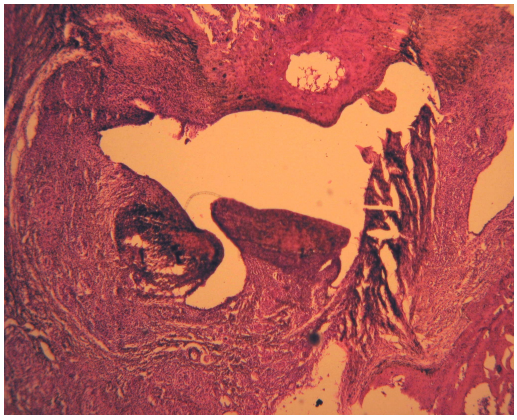


Figure-13: (Group-II) CFA Induced

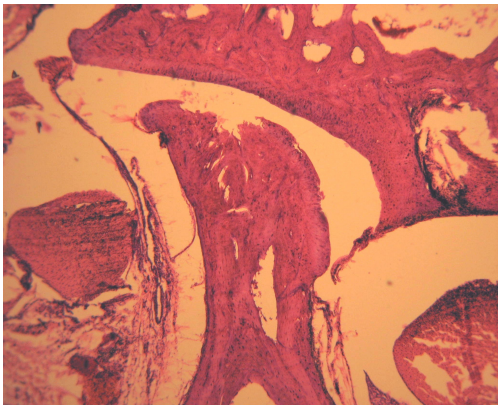


Figure-14: (Group-IV) CFA induced and GF extract treated (200mg/kg)

Plate II: X-RAY STUDY OF RAT FOOT REGION IN CONTROL AND EXPERIMENTAL ANIMALS

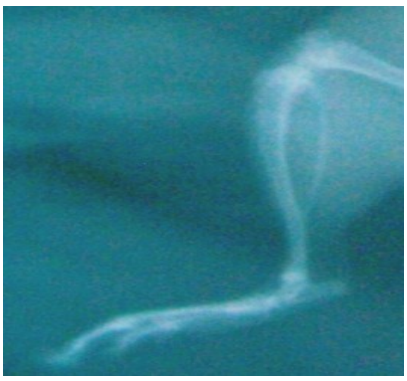


Figure-15: (Group-I) Control

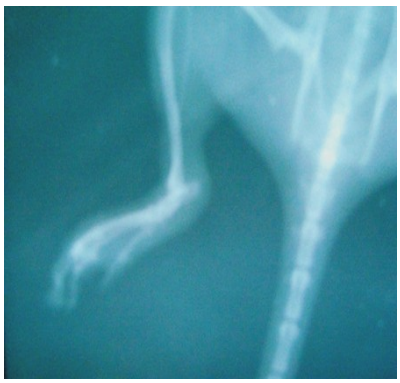


Figure-16: (Group-II) CFA Induced

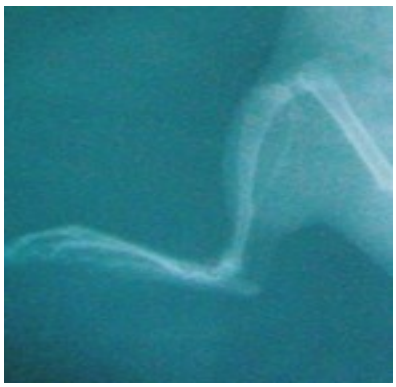


Figure-17: (Group-IV) GF Extract Alone

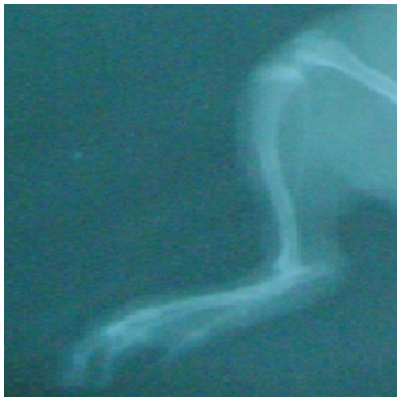


Figure-18: CFA Induced and GF Extract Treated (200mg/kg)

BIBLIOGRAPHY

1. Abuja PM, Albertini R (2001). Methods for monitoring oxidative stress, lipid peroxidation, and oxidation resistance of lipoproteins. *Clin Chim Acta*. 306:1-17.

2. Antioxidants and risk of rheumatoid arthritis. *Ann Rheum Dis.* 53: 51-53

3. Arunachalam G, Chattopadhyay D, Chatterjee S, Mandal AB, Sur TK, Mandal SC (2002). Evaluation of anti-inflammatory activity, of Alstonia macrophylla Wall ex ,A. DC. Leaf extract. *Phytomedicine*. 9(7): 632-635.

4. Baeuerle PA, Henkel T (1994). Function and activation of NF-Kb in the immune systeum. *Annu Rev Immunol.* 12: 141-179.

5. Bandyopadhyay U, Das D, Banerjee RK (1999). Oxidative damage and pathogenesis. *Current Sci*. 77: 515.

6. Barrett AJ, Cathepsin D (1970). Purification of isoenzymes from human and chicken liver. *Biochem. J*. 117: 601–607.

7. Bass D, Lewis A, Szejda JC, Cowley P, McCall CE (1981). Activation of lysosomal acid phosphatase of eosinophils leukocytes. *Lab. Invest.* 44: 403–409.

8. Bazzichi L, Ciompi ML, Betti L (2002). Impaired glutathione reductase activity and level of collogenase and elastase in synovial fluid in rheumatoid arthritis. *Clin Exp Rheumatol*. 20: 761-766.

9. Bazzoni F, Beutler B (1996). The tumor necrosis factor ligand and receptor families. *N. Engl. J. M.* 334: 1717–1725.

10. Beckman JS, Chen J, Ischiropoulos H, Crow JP (1994). Oxidative chemistry of peroxynitrite. *Methods in Enzymology*. 233: 229-240.

11. Besson JM, Guilbaud G (1988). The arthritic a rat as a model of clinical pain In: International congress Series 837. *Exceprta Medica Elsevier. Amsterdam*. 257.

12. Blake DR, Hall ND, Treby DA, Halliwell B, Gutteridge JMC (1981). Protection against superoxide and hydrogen peroxide in synovial fluid from rheumatoid patients. *Clinical Science.* 61: 483-486.

13. Blake DR, Lunce J (1985). *Br J Rheumatol.* 24(2): 123-5.

14. Bombara MP, Webb DL, Conrad P (1993). Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. *J Leukoc Biol. 54*: 399-406.

15. Bouic PJD (1999). Plant sterols and sterolins, a review of their immune-modulating properties. *Altern Med Rev.* 4: 170-177.

16. Buettner GR, Schafer FQ (2000). Free radical, Oxidant, and antioxidant. *Teratology.* 62:234.

17. Carteron NL (2000). Cytokines in rheumatoid arthritis, trials, and tribulations. *Molecular Medicines Today.* 6: 315–323.

18. Caster CW, Furlong AM, Carter-Su C (1958). Connective tissue activation, stimulation of glucose transport by connective tissue activation peptide II. *Biochemistry.* 24: 1762–1767.

19. Chattopadhyay D, Arunachalam G, Mandal AB, Mandal SC (2002). Evaluation of antipyretic activity of leaf extracts of Mallotus peltatus (Geist) Muell. Arg. Var acuminatus: A Folk medicine. *Phytomedicine*. 9: 727-730.

20. Chattopadhyay D, Arunachalam G, Sur TK, Bhattacharya SK, Mandal AB (2005). Analgesic and Antiinflammatory Activity of *Alstonia macrophylla* and *Mallotus peltatus* leaf extracts: Two popular Ethnomedicines of Onge, A Nigrito Tribes of Little Andaman. *Oriental Pharmacy and Experimental Medicine.* 5(2).

21. Chen F, Castranova V, Shi X, Demers LM (1999). New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clinical Chemistry.* 45: 7-17.

22. Choy EH, Panayi GS (2001). Cytokine pathways and joint inflammation in rheumatoid arthritis. *New England Journal of Medicine*. 344: 907–916.

23. Clancy RM, Rediske J, Tang X, Nijher N, Frenkel S, Philips M, Abramson SB (1997). Outside-in signaling in the chondrocyte. Nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmalactin/rho A/focal adhesion kinase-signaling complex**.** *J Clin Invest.* 100**:** 1789-1796.

24. Davies KJA (1995). Oxidative stress, The Paradox of aerobic life. *Biochem Soc Symp.* 61: 1-31.

25. Dayer JM (2003). The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. *Rheum.* 42: ii3–ii10.

26. De Leo ME, Tranghese A, Passantino M (2002). Manganese superoxide dismutase, glutathione peroxidase, and total radical trapping antioxidation capacity in active rheumatoid arthritis. *J Rheumatol*. 29: 2245-2246.

27. Debets JM, Vander Linden CJ, Dieteren IE, Leeuwenberg JF, Buurman WA (1988). Fc-receptor cross-linking induces rapid secretion of tumor necrosis factor (cachectin) by human peripheral blood monocytes. *J Immunol.* 141: 1197-201.

28. Debets JM, Vander Linden CJ, Dieteren IE, Leeuwenberg JF, Buurman WA (1988). Fc-receptor cross-linking induces rapid secretion of tumor necrosis factor (cachectin) by human peripheral blood monocytes. *J Immunol.* 141: 1197-201.

29. Diment S, Martin KJ, Stahl PD (1989). Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. *J. Biol.Chem*. 264: 13403–13406.

30. Dröge W (2002). Free Radicals in the Physiological Control of Cell Function. *Physiol Rev.* 82: 47–95.

31. Falkenbach A, Unkelbach U, Gottschalk R, Kaltwasser JP (1991). Serum level of beta-glucuronidase as potential indicator of disease activity in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). *Med. Klin. (Munich).* 86: 465–468.

32. Feldmann M, Brennan FM, Maini RN (1996). Role of cytokines in rheumatoid arthritis**.** *Annu Rev Immunol.* 14: 397–440.

33. Fleischmann R, Stern R, Iqbal Anakinra I (2004). An inhibitor of IL-1for the treatment of rheumatoid arthritis. *Expert Opinion on Biol Thera*. 4: 1333–1344.

34. Flohe L, Beckmann R, Giertz H (1985). Oxygen-centered free radicals as mediators of inflammation, In: Sies H, editor. Oxidative stress. *London Academic Press*. 403-436.

35. Fox DA (2005). Etiology and pathogenesis of rheumatoid arthritis, In: Koopman WJ, Moreland LW, eds. *Arthritis and allied conditions a textbook of rheumatology, Fifteenth edition. Philadelphia; Lippincot Williams and Wilkins;: 1089 - 1115***.**

36. Frauwirth KA, Thompson CB (2002). Activation and inhibition of lymphocytes by costimulation. *J Clin Invest.* 109: 295-99.

37. Geetha T, Varalaxmi P, Latha RM (1999). Effect of lupeol & lupeol linoleate on lysosomal enzymes & collagen in adjuvant induced arthritis in rats. *Mol. and Cell Biochem.* 201: 83-87.

38. Ghiselli A, Serafini M, Natella F, Scaccini C (2000). Total antioxidant capacity as a tool to assess redox status:critical view and experimental data. *Free Radic Biol Med*, 29: 1106-1114.

39. Guardia T, Rotelli AE, Juarez AO, Pelzer LE (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco*. 56: 683–687.

40. Gupta MB, Nath R, Srivastava N (1996). Antiinflammatory and antipyretic activities of beta sitosterol. *Internat J Immunopharmacol.* 18: 693-700.

41. Gutteridge JMC (1986). Antioxidant properties of the protein ceruloplasmin, albumin and transferring; A study of the activity in serum and synovial fluid from patients with rheumatoid arthritis. *Biochem Biophys: Acta.* 869: 119–127.

42. Han SK, Ko YI, Park SJ, Jin IJ, Kim JM (1997): Oleanolic acid and ursolic acid stabilize liposomal membranes. *Lipids* 32: 769-773.

43. Hasilik A, Neufeld EF (1980). Biosynthesis of lysosomal enzymes in fibroblasts, Synthesis as precursors of higher molecular weight. *J. Biol. Chem*. 255: 4937–4945.

44. Heliovaara M, Knekt P, Aho K, Aaran RK, Alfthan G, Aromaa A (1994). Serum.

45. Henderson B, Pettipher ER (1989). Arthritogenic actions of recombinant IL-1 and tumour necrosis factor alpha in the rabbit: evidence for synergistic interactions between cytokines in vivo**.** *Clin Exp Immunol*. 75: 306–310.

46. Henrotin YE, Bruckner P, Pujol JP (2003). The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis Cartilage*. 11**:** 747-755.

47. Ischiropoulos H, Zhu L, Chen J, Tsai, Martin J, Smith C, Beckman, JS (1992). Peroxynitrite mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys*. 298: 431– 437.

48. Jackson DS, Bentley JP (1968). In: Treatise in Collagen. *Academic Press (B.S.Gould ed.), London*. 189-214.

49. Jagetia GC, Baliga MS, Malagi KJ, Sethukumar Kamath M (2002). The evaluation of the radioprotective effect of Triphala, (an ayurvedic rejuvenating drug) in the mice exposed to γ-radiation. *Phytomedicine*. 9: 99–108.

50. Jasin HE (2005). Mechanisms of tissue damage in rheumatoid arthritis. In: Koopman WJ, Moreland LW, eds. Arthritis and allied conditions a textbook of rheumatology; Fifteenth edition. *Philadelphia. Lippincot Williams and Wilkins*. 1141-1164.

51. Jaswal S, Metha HC, Sood AK, Kaur J (2009). Antioxidant status in rheumatoid arthritis and role of antioxidant therapy. *Clin chim ACTA*. 338: 123-129.

52. Kamanli A, Naziroglu M, Aydile K (2004). Plasma lipid peroxidation, and antioxidant levels in patients with rheumatoid arthritis. *Cell Biochem Funct*. 22: 53-57.

53. Karatas F, Ozates I, Canatan H (2003). Antioxidant status and lipid peroxidation in patients witrheumatoid arthritis. *Indian J Med Res*. 118:178-181.

54. Kawai Y and Anno K (1971). Mucopolysaccharide - degrading enzymes from the liver of squid Ommastrephes solani pacificus. I. Hyaluronidase. *Biochim Biophys Acta* 242: 428-436

55. King J (1965a). The hydrolases-acid and alkaline phosphatases. In: *Practical clinical enzymology* (King JC, ed.), Van D Nostrand Company, London pp.199-208

56. Koch AE (1998). Review: angiogenesis, implications for rheumatoid arthritis**.** *Arthritis Rheum.* 41: 951–962.

57. Kong YY, Feige U, Sarosi I (1999). Activated t cells regulate bone loss and joint destruction in adjuvant arthritis though osteoprotegerin ligand. *Nature.* 402: 304-09.

58. Levinson SS (1994). Humoral mechanisms in autoimmune disease. *J Clin Immunoassay.* 17: 72-84.

59. Lotz M(2003). Neuropeptides free radicals and nitric oxide; In *Rheumatology*; 3rd edition, Edited by Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH; Toronto: Mosby;:135-146.

60. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.

61. Lunec J, Halloran SP, White AG, Dormandyee TL (1981). Free radical oxidation Peroxidation products in serum and synovial fluid in rheumatoid arthritis. *J. Rheumatol*. 8: 233-4.

62. Mangge H, Gindl S, Kenzian H, Schauenstein K (2003). Atopic dermatitis as a side effect of anti-tumor necrosis factor-alpha therapy. *Journal of Rheumatology.* 30: 2506–2507.

63. Marnett LJ (1999). Lipid peroxidation-DNA damage malonaldialdehyde. *Mutat Res.* 424: 83-95.

64. Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, Habermehl GG (1989). *Biochem. J.* 263**:** 539–545.

65. Miesel R, Hartung R & Kroeger H (1996a) Priming of NADPH oxidase by tumor necrosis factor alpha in patients with inflammatory and autoimmune rheumatic diseases. *Inflammation.* 20: 427-438.

66. Miesel R, Kurpisz M, Kroger H (1996). Suppression ofinflammatory arthritis by simultaneous inhibition of nitric oxide synthase and NADPH oxidase. *Free Radical Biology and Medicine.* 20: 75-81.

67. Mittal N, Kaur J, Mahmood A (1996). Changes in tubular membrane glycosylation in diabetic insulin and thyroxin-treated rat kidneys. *Indian Journal of Experimental Biology*. 34: 782–785.

68. Moore JC and Morris JE (1982). A simple automated colorimetric method for determination of N-acetyl-beta-D-glucosaminidase. *Ann Clin Biochem* 19: 157-159

69. Morel Y, Baroiki R (1998). Gene regulation by oxidative stress. *Medicine/Sciences*. 14: 713-721.

70. Narendhirakannan a RT, Subramanian b S, Kandaswamy a M (2007). Anti-inflammatory and lysosomal stability actions ofCleome gynandra L. *studied in adjuvant induced arthritic rats Food and Chemical Toxicology*. 45: 1001–1012.

71. Neibes P (1972). Determination of enzymes and degradation products of glycosaminoglycan metabolism in healthy and various subjects. *Clin Chem Acta* 42: 399-408

72. Newton RC, Decicco CP (1999). Therapeutic potential and strategies for inhibiting tumor necrosis factor-alpha. *J. Med, Chem*. 42: 2295–2314.

73. Nurcombe HL, Bucknall RC, Edwards SW (1991). Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: priming and activation in vivo. *Annals of Rheumatic Diseases.* 50: 147-153.

74. O'Reilly MS, Boehm T, Shing Y (1997). Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 88: 277-85.

75. Ortiz E (2004). Market withdrawal of Vioxx: is it time to rethink the use of COX-2 inhibitors?. *Journal of Managed Care Pharmacy.* 10: 551–554.

76. Page C, Curtis MJ, Sutter MC, Walker MJA, Hoffman BB (2002). *Integrated Pharmacology, 2nd ed, USA: Mosby Inc*.

77. Perkins DJ, St Clair EW, Misukonis MA, Weinberg JB (1998). Reduction of NOS2 overexpression in rheumatoid arthritis patients treated with anti-tumour necrosis factor a monoclonal antibody (cA2). *Arthritis and Rheumatism*. 41: 2205-2210.

78. Pillinger MH, Abramson SB (1995). The neutrophil in rheumatoid arthritis. *Rheum Dis Clin North Am.* 21: 691-714. 115.

79. Probert L, Plows D, Kontogeorgos G, Kollias G (1995). The type I interleukin- 1 receptor acts in series with tumor necrosis factor (TNF) to induce arthritis in TNF-transgenic mice**.** *Eur J Immunol*. 25:1794–1797.

80. Pryor WA, Squadrito GL (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am J Physio.* 268: L699–L722.

81. Rainsford KD (1982). Adjuvant polyarthritis in rats: is this a satisfactory model for screening arthritic drugs? . *Agents Actions.* 12: 452-458.

82. Ralston SH, Grabowski PS (1996). Mechanisms of cytokine induced bone resorption: role of nitric oxide, cyclic guanosine monophosphate and prostaglandins. *Bone*. 19: 29-33.

83. Ravichandran LV, Puvanakrishnan R, Joseph KT (1990). Alterations in heart lysosomal stability in isoproterenol induced myocardial infarction in rats. *Biochemical Interactions.* 22: 387–396.

84. Rediske JJ, Koehne CF, Zhang B, Lotz M (1994). The inducible production of nitric oxide by articular cell types. *Osteoarthritis and Cartilage*. 2: 199-206.

85. Richo G, Conner GE (1991). Proteolytic activation of human procathepsin D. *Adv. Exp. Med. Biol*. 306: 289–296.

86. Richo G, Conner GE (1994). Structural requirements of procathepsin D activation and maturation. *J. Biol. Chem*. 269: 14806–14812.

87. Safina A, Korolenco T, Mynkina G, Dushkin M, Krasnoselskaya G (1992). Liver and serum lysosomal enzyme activity during zymosan induced inflammation in mice. *Agents Actions*. 38: 370-375.

88. Sakurai HG, Kohsaka H, Liu M-F, Higashiyama H, Hirata Y, Kanno K, Saito I, Iyasaka N (1995). Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *Journal of Clinical Investigation.* 96: 2357-2363.

89. Sapolsky AL, Altman RD and Howell DS (1973). Cathepsin D activity in normal and osteoarthritic human cartilage. *Fed Proc* 32: 1489-1493.

90. Schuna AA, Megeff C (2000). New drugs for the treatment of rheumatoid arthritis. *Am, J. Health-Syst, Pharm*. 57: 225–237.

91. Shanmugarajan TS, Niladri M, Somasundaram I, Prithwish Soncharan Patel N, Nazeer Ahamed KFH (2008). Evaluation of Antioxidant Potential of *Gaultheria fragrantissima* Wall. *Against Adjuvant Induced Arthritis in Wistar Rats.*

92. Silva JC, Rocha MF, Lima AA, Brito GA, de Menezes DB, Rao VS (2000). Effects of pentoxifylline and nabumetone on the serum levels of IL-1b and TNFa in rats with adjuvant arthritis. *Inflamm Res*. 49: 14–19.

93. Smolen S Josef, Daniel Aletaha, Marcus Koeller, Michael H Weisman, Paul Emery (2007). New therapies for treatment of rheumatoid arthritis. *New Drug Class.*

94. Steiner G, Smolen JS (2006). Autoantibodies in rheumatoid arthritis, In : Firestein GS, Panayi GS, Wollheim FA. Eds rheumatoid arthritis, 2nd edn. *Oxford: Oxford University Press.* 194-98.

95. Stichtenoth DO, Frolich JC (1998). Nitric oxide and inflammatory joint diseases. *British Journal of Rheumatology.* 37: 246-257.

96. Surinder Kumar, Yogeeta, Arunachalam, Gnanapragasam, Subramanian, Senthilkumar, Rajakannu, Subhashini, Thiruvengadam, Devaki (2006). Synergistic salubrious effect of ferulic acid and ascorbic acid on membrane-bound phosphatases and lysosomal hydrolases during experimental myocardial infarction in rats. *Life Sciences.* 80: 258–263.

97. Tiku ML, Liesch JB, Robertson FM (1990). Production of hydrogen peroxide by rabbit articular chondrocytes, Enhancement by cytokines. *Journal of Immunology*. 145: 690-696.

98. Tripathy AK, Shukla YN, Kumar S (1996). Ashwagandha (Withania somnifera) Dunal (Solanaceae), A status report, Med. Arom. *Plant. Sci*. 18: 46–62.

99. Tsuji G, Koshiba M, Nakamura H, Kosaka H, Hatachi S, Kurimoto C, Kurosaka M, Hayashi Y, Yodoi J, Kumagai S (2006). Thioredoxin protects against joint destruction in a murine arthritis model. *Free Radic. Biol. Med*. 40: 1721–1731.

100. Wagner WD (1979). A more sensitive assay discriminating galactosamine and glucosamine in mixtures. *Anal Biochem* 94: 394-396.

101. Warren L (1959). The thiobarbituric acid assay of sialic acids. *J Biol Chem* 234: 1971-1975

102. Wattiaux (1977). In: *Mamalian cell membranes* (Jamieson GA and Robinson DM, ed.), Vol.2, Butterworth Press, London, pp.165-177

103. Weller PF, Austen KF (1983). Human eosinophil arylsulfatase B. Structure and activity of the purified tetrameric lysosomal hydrolases. *J. Clin. Invest.* 71**:** 114–123.

104. Wood NC, Symons JA, Dickens E, Duff GW (1992). In situ hybridization of IL-6 in rheumatoid arthritis. *Clin Exp Immunol*. 87: 183–189.

105. Yasuda M, Okabe T, Itoth J, Takekoshi S, Hasegawa H, Nagata H, Osamura Y, Watanabe K (2000). Differentiation of necrotic cell death with or without lysosomal activation, Application of acute liver injury models induced by CCl4 and dimethylnitrosamine. *Journal of Histochemistry and Cytochemistry*. 48: 1331–1339.