Analyzing Anti-Arthritic Effect of Hydrogels Embedded with *Thymus serpyllum* Extract Through In-Vivo Study

Misha Zaib

Reg. No. 00000329678

Supervised by

Prof. Dr. Peter John

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST),

Islamabad, Pakistan.

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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THESIS ACCEPTANCE CERTIFICATE

Certified that the contents and form of thesis entitled "**Analyzing anti-arthritic effect of hydrogels embedded with *Thymus serpyllum* extract through In-vivo study** "submitted by **Misha Zaib** has been found satisfactory for requirement of degree.

Supervisor: _____

Prof. Dr. Peter John

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Head of Department: _____

Dr. Saadia Zahid

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Principal: _____

Dr. Hussnain Janjua

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Dated: _____

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Signature of Student

Misha Zaib

00000329678

DEDICATION

This dissertation is dedicated to my parents, **Muhammad Zaib** and **Zeenat Zaib**, who instilled in me the virtue of perseverance and commitment. I would like to thank them for believing in me and encouraging me relentlessly to achieve my dreams.

I would also like to dedicate this dissertation to my siblings for supporting me throughout this journey.

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Misha Zaib.

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LIST OF ACRONYMS

ACPA	Anti-citrullinated peptide antibody
CIA	Collagen induced arthritis
CRP	C-reactive protein
ESR	Erythrocyte sedimentation rate
HLA	Human leukocyte antigen
IL-6	Interleukin-6
ME	Microemulsion
O/W	Oil in Water
PBS	Phosphate Buffer Saline
PDI	Polydispersity index
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
SE	Shared epitope
Smix	Surfactant Co-Surfactant mixture
Th1	T-helper cell 1
TNF-alpha	Tumor Necrosis Factor- alpha
W/O	Water in Oil

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ABSTRACT

Rheumatoid arthritis is an autoimmune disease that effects 1% of population worldwide. Several genetic and environmental factors play a role in the onset of the disease. The HLA-DRB1 locus is the most studied genetic factor which produces a 5 amino acids molecule known as the shared epitope which is currently used as a diagnostic marker. In addition to the genetic, the environmental factors like smoke, dust and even the microbiome also play a role in its pathogenesis. There is currently no cure for rheumatoid arthritis but different drugs are used to manage its symptoms and stop its prognosis. These drugs provide therapeutic effect to a limit and their prolong use is associated with numerous side effects like stomach ulcers, kidney failure, heart diseases etc. Microemulsion is a new drug delivery system that helps to minimize the toxic effects of drugs by providing a targeted release and improve their efficacy by increasing their permeability. The topical route of administration of microemulsions increases the bioavailability of the active compound by stopping the drug from getting degraded by the enzymes. Due to the favorable outcomes of topical based microemulsion systems, they are emerging as a promising drug carrier contributing to the therapeutic evolution as they not only improve the patient's life by suppressing the symptoms but they also medicate the inflammatory processes.

Thymus serpyllum is being used as an herbal medicine in the form of tinctures, syrups, oil and tea for centuries. The upper parts of thyme are used as an antiseptic, anthelmintic, disinfectant, antispasmodic, sedative and expectorant. *T. serpyllum* is commonly used against disorders concerning gastrointestinal and respiratory system. Carvacrol is the major plant oil constituent that shows *in vitro* cytotoxic activity against the cancer cell lines. Thyme oil shows a concentration dependent viability of cell lines.

The ethanolic extract showed positive results for various bioactive phytochemicals like phenols, flavonoids, Saponins, steroids, anthraquinones and glycosides. Extract exhibit good anti-oxidant activity assessed by DPPH assay and FRAP assay. It also displayed significant anti-inflammatory activity assessed by Percentage inhibition of albumin denaturation assay and membrane stabilization assay. Plant extract showed no toxic effects in in-vivo and ex-vivo as confirmed by acute toxicity (LD50) and hemolytic assays.

Abstract

Microemulsion was prepared by using oleic acid as oil component, tween 20 as surfactant and ethanol as co-surfactant. The constituent ratios of components were determined by the pseudo-ternary phase diagrams. The formulated microemulsion was characterized for size, zeta potential, PDI and pH. Stability of the microemulsion was also confirmed by the use of centrifugation, dilution tests and heat and cycles.

The anti-arthritic potential of the microemulsion was confirmed by the use of collagen induced arthritis BALB/c mouse models. The formulations showed significant decrease in paw measurements and the X-rays showed that the treated groups had less inflammation and joint deformation as compared to the control groups. The group treated with plant based microemulsion showed serum levels within the normal range.

The current study concluded the anti-arthritic effect of Thymus serpyllum embedded microemulsions in in-vivo studies.

CHAPTER 1
INTRODUCTION

1.1 Autoimmunity

Immune system is involved with function of detecting foreign antigens like toxins or infectious agents, and extirpating them. Lymphocytes are the major cells of immune system that detect and either directly attacks the foreign antigens or produce antibodies against them. With the presence of a large variety of immune cells in the blood circulation, it is inevitable that some cells would react to self-antigens. These are body's own healthy cells which are detected as foreign antigens by the immune system. This state is referred to as autoimmunity, the condition in which the immune system responds to the body's own healthy components, leading to different autoimmune diseases and functional changes (Britannica, 2018). Autoimmunity is caused by the same response of adaptive immune system directed towards an infection.

Like all adaptive immune responses, the autoimmune disease occurs when specific self-antigens are recognized by T-cell and B-cell receptors. These self-antigens are actually proteins produced by the organs which triggers a chronic inflammatory response disrupting the normal functioning of the tissue. The autoimmune response can be organ-specific i.e. a localized response affecting a single organ or tissue, or systemic i.e. a general inflammatory response affecting several organs. The trigger for autoimmune disorders is usually hard to determine as several factors could be involved. An infection can also initiate the process of autoimmunity as the antigens released by the infectious agents might be similar to the body's own proteins. Other environmental factors such as smoke, dust and pollen might also be related to the onset of autoimmunity. Studies show that autoimmune diseases might also be inherited. A number of polymorphic genes influence the susceptibility to autoimmune diseases. These genes alone have very little effect but influence greatly when aggregated (Nicholson).

1.1.1 Gender and Autoimmunity

Autoimmune diseases are multifactorial diseases depending on both intrinsic and extrinsic factors. The intrinsic factors show diverse genetic polymorphisms signifying a mosaic of autoimmunity where the immune response is different for each patient. Clinical and experimental data suggests the influence of gender on autoimmunity. Females have enhanced immune reactivity than males, showing an increased activity of antigen presentation of lymphocytes and monocytes and a higher mitogenic response. Females show higher levels of immunoglobulins and have increased antibody production than males. Due this increased immune reactivity, females have a higher homograft rejection rate as males whereas, males are more prone to infections. This data indicates the influence of sex hormones on gender bias in autoimmunity.

Sex hormones play a role in controlling different immune system functions i.e. the maturation and activation of lymphocytes, synthesis of autoantibodies and pro-inflammatory cytokines. This kind of dimorphism is also observed in patients of rheumatoid arthritis (RA). Studies show RA patients have low androgens and progesterone levels, and male patients particularly have decreased levels of testosterone, dehydroepiandrosterone (DHEA) and estrone. In contrast to this, these male patients have increases levels of estradiol, an estrogen steroid hormone, that correlates with the inflammatory indices (Gisele Zandman-Goddard, 2007).

1.2 Rheumatoid Arthritis

In an autoimmune disease, the immune system mistakes the body's own cells as foreign invaders and attacks them. It can affect one organ or multiple parts of the body by producing autoantibodies. One such example of autoimmune disease that effect the joints is rheumatoid arthritis. (Watson, 2019)

RA is a type of chronic inflammatory disease that symmetrically effects the joints. It initially effects small joints like the wrists, slowly progressing to larger joints and eventually targeting organs like eyes, heart, skin and lungs. It causes erosive synovitis subsequent to the destruction of cartilage and bones at the joints resulting in swelling and immobility (Bullock J, 2018). It is the most common type of inflammatory arthritis, with

an annual rate of 1% prevalence and 3 per 10,000 cases incidence worldwide. Unlike osteoarthritis it has an early onset of around 30 – 50 years of age. (Smith, 2022) Women are affected two to three times more than men suggesting a link between the disease and the sex hormones. According to expert's opinion, the female immune system is more reactive and stronger than the male due to which they are more susceptible to autoimmune diseases. It is also believed that the hormones entail RA risk and flares (Why Women Get More Arthritis Than Men, 2017). A study in Denmark showed that the rate of RA was higher in females who had 1 offspring than to those with 2 – 3, as pregnancy improves RA due to the shift in hormones. There was no change in the rate of nulliparous females or those with a history of miscarriage (Brown, 2021). Another study also exhibited breastfeeding as a way to reduce the risk of RA as it was proved that breastfeeding for 2 or more years reduced the probability of the disease by half (Brunilda Nazario, 2020).

Currently there is no cure for rheumatoid arthritis but the symptoms and progression of the disease can be managed by making the patients aware about the disease, through conducting physical therapy and treatment through various drugs. The drug treatment approaches consist of non-steroidal anti-inflammatory drugs and glucocorticoids, disease modifying anti-rheumatic drugs and biologics (Vikas Majithia, 2007). These traditional drug treatments have been reported to show limited efficacy and cause adverse side effects hence there is a need to develop new approaches for targeted and efficient drug delivery to minimize these limitations.

1.3 Epidemiology of Rheumatoid Arthritis

According to the Global Burden of Disease 2010 Study, the worldwide RA prevalence is around 0.24 percent, with a higher estimated prevalence of 0.5 and 1 percent in the United States (U.S) and other northern European countries. The annual incidence rate in these countries is almost 40 per 100,000 individuals. Most of the available data on RA comes from US and northern European countries due to majority of studies been conducted in these regions (Bryant R England, 2022). Between the years 1980 and 2019, reported global prevalence of RA was 460 patients per 100,000 individuals. The variation is mostly due to a difference in study methodology and the geographical locations of the reported data (Khalid Almutairi, 2021).

In Pakistan, the point prevalence of RA was reported as 26.9 percent of patients in Karachi with a mean age of 44 years. Another study conducted also show cased the functional disability that come with the onset of Rheumatoid Arthritis and their significant correlation with depression (Atta Abbas Naqvi, 2019)

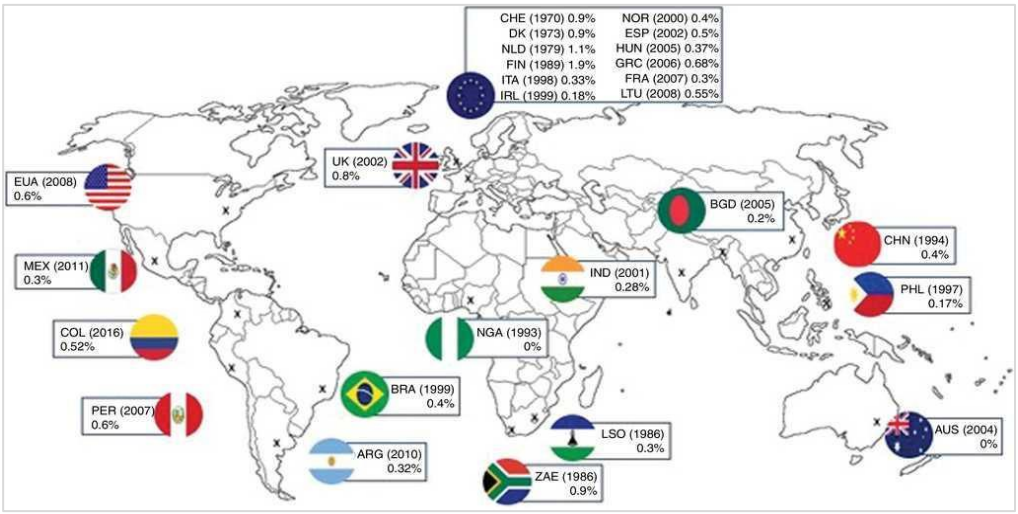

Figure 1: *Prevalence of Rheumatoid Arthritis across the globe (Fernández-Ávila, 2019)*

1.4 Diagnosis of Rheumatoid Arthritis

Rheumatoid arthritis causes inflammation of the synovium (synovial membrane), which is an integral part of the synovial joints as it lines the inner surfaces. The hallmark of RA is the pannus formation which is formed due to the proliferation of synovial tissues. It harbors a large number of immunological cells like fibroblasts which perpetuates inflammation and damage the articular structures like bone and cartilage causing synovial hypertrophy (Luis Javier Cajas, 2019). RA is an autoimmune disease believed to be caused by an infection as its exact cause is still unknown. Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are considered as the main mediators of the disease as they trigger the immune system to produce autoantibodies. RA is diagnosed on the basis of patient's clinical features, X-ray images and detection of the serological markers. The serological markers are the autoantibodies produced in the case of RA. The most common autoantibody is known as rheumatoid factor (RF). The presence and absence of RF can determine which type of rheumatoid arthritis is present in the body. Another important marker of RA is the anti-

cyclic citrullinated peptide antibody (ACPA), which is more sensitive and specific than RF in determining the diagnosis and prognosis of the disease (Sang-Tae Choi, 2018).

1.4.1 Blood Tests

The diagnosis cannot be done on the basis of a single blood test hence multiple tests are conducted to indicate that RA is the actual source of the present symptoms. Following are a few of the blood tests that are conducted to confirm the diagnosis:

1.4.1.1 Erythrocytes Sedimentation Rate (ESR) test:

During inflammation the red blood cells aggregate together which causes them to separate from the rest of the blood cells much faster. An ESR test measures how quickly the red blood cells (erythrocytes) separate and accumulate at the bottom of the collection tubes. A higher ESR level indicates presence of inflammation and a lower ESR level shows no or low inflammation. This test alone is not enough to diagnose RA as a higher ESR level and inflammation can be also be a cause of chronic infections.

The sedimentation rates vary with gender and age, tending to be higher for people with older ages (< 50 years). A normal range for men is 0 - 20 mm per hour and for women its 0 – 30 mm per hour (Nall, 2021).

1.4.1.2 C- Reactive Protein (CRP) test:

CRP is produced by the liver during an infection in our body. This protein triggers the immune system to respond towards the infectious agents causing inflammation through the body. The CRP test measures the amount of CRP present in the bloodstream.

In an autoimmune disease, the body mistakes its own cells as foreign bodies and incite the immune cells to destroy them. Hence in an autoimmune disease like Rheumatoid Arthritis, there are high levels C-reactive protein which indicates high levels of inflammation. Levels above 10mg/L suggests significant inflammation (Kandola, 2022).

1.4.1.3 Complete Blood Count (CBC):

This test is use to evaluate the levels of different cells present in the blood i.e., red blood cells, white blood cells and platelets. RA does not disrupt the blood cells but different

conditions having similar symptoms like RA can cause the CBC test to have abnormal results.

Anemia is a condition associated with lack of red blood cells and is common in people with RA but is not directly linked to RA (Diagnosis, 2019).

1.4.1.4 Antibody tests:

The presence of RF and ACPA antibodies can help indicate the diagnosis for RA. RF is an antibody produced against the gamma globulins. A higher level of RF than normal range (0-20units/ml) points towards RA but it is not always correct, as in 5% of cases higher levels are linked with other conditions like leukemia, lupus and hepatitis. Some people with RA also test negative for RF antibody test.

An ACPA test is used to diagnose and determine the severity of RA. About 60% to 80% of RA patients are positive for this autoantibody. Usually a doctor conducts both RF and ACPA tests to evaluate RA (Blood Tests for Rheumatoid Arthritis, 2016).

TEST RESULTS	***RESULT INTERPRETATION***
Positive RF and Positive ACPA	Higher risk for RA
Negative RF and Positive ACPA	Early stage RA or likely to develop RA
Negative RF and Negative ACPA	Less likely to develop RA

Table 1: *Result interpretation of RF and ACPA test*

1.4.2 Imaging tests

Imaging tests are not diagnostic but they are used to support the findings obtained from physical examinations and blood tests. Since early times X-rays have been used to diagnose RA but they fail to show any abnormality during the early stages of disease and are not able to timely diagnose RA which is very critical for the management of the disease. With the advancement in science, newer imaging technologies like musculoskeletal ultrasound (MSUS) and magnetic resonance imaging (MRI) are used which are able to detect inflammation and bone erosion during the initial phase of RA helping in early treatment to reduce joint damage (Imaging Tests for Rheumatoid Arthritis, 2022).

1.5 Types of Rheumatoid Arthritis

1.5.1 Seropositive Rheumatoid Arthritis

Two third of the RA population is designated as seropositive as they are tested positive for the presence of RF and/or ACPA. ACPAs and other inflammatory cytokines can be identified 2-4 years before the clinical onset of the disease (Reed, 2020). Citrullinated peptides activate the T cells by playing a role as an autoantigen that is recognized by T-cells to produce a pro-inflammatory environment that damages the joints. This initiates the B-cells to produce the autoantibodies that causes bone erosion.

1.5.2 Seronegative Rheumatoid Arthritis

The seronegative patients show negative results for the presence of RF and/or ACPA. The initial phase of the disease is more vigorous in these patients. They also show a lagging response to the treatments as compared to a seropositive patient. The seropositive and seronegative RA is differently characterized on the basis of genetic and environmental factors, preclinical and clinical phases and the cytokines present in the synovial fluid.

A new type of peptide is also being recognized as a potential biomarker for the seronegative RA as they lack the presence of RF and ACPAs. It is identified in seropositive population as well as seronegative but not in the healthy population due to its higher epitope specificity. Hence it can be used as a novel serum biomarker for the seronegative RA (Bason Caterina, 2021).

1.6 Etiology of Rheumatoid Arthritis

Since early times, genetic and epigenetic factors have been associated with the onset of RA. With recent studies, environmental factors have also been associated with the inception of the disease. Exposure to air pollutants like dust and cigarette smoke can trigger the inflammatory process by activating the immune system. The internal microbiota of a person, that helps to maintain the homeostasis condition of a body, can also be involved in the commencement of the disease.

1.6.1 Genetic Factors

Genetic predisposition plays an important role in the development and severity of the disease. A number of single nucleotide polymorphisms (SNPs) of specific genes have been associated with RA. They are not unique to RA but are also involved in susceptibility to other autoimmune diseases. In a study on 148 identical twins, it was revealed that the heritability of RA was 66% showcasing that the genetic predisposition does play a role in the pathogenesis of the disease (D. van der Woude, 2009). This does underline the importance of genetic loci in disease susceptibility but it also indicates the involvement of other environmental factors.

One of the most conventional genetic predispositions is the human leukocyte antigen (HLA) class II on the chromosome 6 which is associated with many autoimmune diseases. For RA it is the inheritance of particular HLA haplotypes, characterized by the alleles at the HLA-DRB1 locus, particularly the HLA-DR4 and HLA-DR1. These haplotypes produce a specific molecule with a five amino acid motif known as the "shared epitope (SE)" that codes for the positively charged P4 peptide bonding pocket (P.K. Gregersen, 1987). HLA-DR4 is associated with a severe case of RA, causing the joint and bone destruction specially when the person is homozygous for the allele. The SE-bearing HLA-DRB1 haplotypes are identified in the seropositive or ACPA- positive RA and hence can be used as identification markers. For seronegative or ACPA negative RA, the non-SE-bearing HLA alleles like HLA-DR3 are identified as the genetic risk factors. The SE-bearing HLA-DR present a negatively charged or a neutral self-peptide that binds with the positive P4-binding pocket causing CD4 T cell activation (Hans Ulrich Scherer, 2020).

Similarly, PTPN22, FcγRs, CTLA4, PADI-4 and other cytokines/cytokines receptors have been associated with RA in different populations.

1.6.2 Environmental Factors

Environmental factors like smoking and dust, lead to many autoimmune diseases including RA by the giving rise to epigenetic modifications. Smoking can increase the risk of RA 21 times for the homozygous HLA-DR SE allele compared to the nonsmokers with no SE genes. This shows a link between a specific genotype and an environmental factor in the onset of a specific subset of RA. (ACPA positive RA) (L. Klareskog, 2006). The

environmental stress, like smoking, causes the posttranslational modification of the proteins. One such protein is the mucosal protein which is citrullinated by peptidyl arginine deiminase type IV (PADI4), stimulating the production of autoantibodies. Around 44% to 64% of ACPA positive RA patients are also positive for citrullinated α-enolase, showing a strong correlation between smoking and the genetic factors like HLA-DRB1 and PTPN22 (Mahdi, 2009).

1.6.3 Microbiome Factors

Infectious agents, mostly the bacterial microorganisms like E. coli, EBV and cytomegalovirus etc., and their products have been linked with the risk of RA as the sequence similarities between the self and foreign pathogen derived peptides can cause the activation of autoreactive T or B cells. This is known as the molecular mimicry. Bacterial infections may cause immune response that form the autoantibody known as the RF factor. This autoantibody targets the Fc portion of IgE or IgG resulting in an autoimmune response.

The gut microbiota and other manageable bacteria like Proteus mirabilis and Klebsiella pneumonia, influence the progression of autoimmunity and is associated with seropositive RA (Taneja, 2014).

1.7 Pathogenesis of Rheumatoid Arthritis

The innate immune response is involved in the pathogenesis of RA. In the innate immune response, the exogenous and/or autologous antigens activate the dendritic cells which then triggers the adaptive immune system by the activation of T cells. In RA, the antigen-presenting cells (APCs), like dendritic cells, activated B cells and macrophages, present the arthritis-associated antigens to the class II MHC molecules. As already discussed, about 80% of the RA patients have the HLA-DRB1*04 gene which changes the binding and presentation of arthritis related antigens to T-cell receptors. This leads to the stimulation and expansion of T cells in the joints. T cells in the synovial membrane are upregulated by the secretion of different lymphokines like IL-2 and IFN- γ lymphokines (Choy, 2012). These stimulated T cells activate macrophages, B cells, osteoclasts and fibroblasts, figure 1. The B lymphocytes express the CD20 and CD22 molecules and differentiate into plasma

cells that secrete antibodies, even the autoantibodies to citrullinated peptides, IgG, RA antigens and fibrinogen etc. Presence of autoantibodies is associated with a severe type of RA as it forms an immune complex that produces proinflammatory cytokines.

The synovial membrane of a rheumatic patient harbors the innate and adaptive immune cells. The infiltration of immune cells stimulates the formation of new blood vessels (neovascularization) which causes the upregulation of fibroblast-like synoviocytes and inflammatory mediators. Prostaglandins and matrix metalloproteinases are the inflammatory mediators produced at the site of injury. Matrix metalloproteinases causes the fibroblast like synoviocytes to destroy bone and joint cartilage. Bone erosion is caused due to the activation of osteoclasts, which are derived from monocytes and macrophages and develop in the synovial membrane.

The macrophage-like cells secrete TNF and interleukin 1 (IL-1), the inflammatory cytokines, into the synovium. Interleukin 6 (IL-6) is another proinflammatory cytokine, that is involved in the pathogenesis of RA. It activates T cells, macrophages and osteoclasts and instigate the production of antibodies by B cells (Josef S Smolen, 2007).

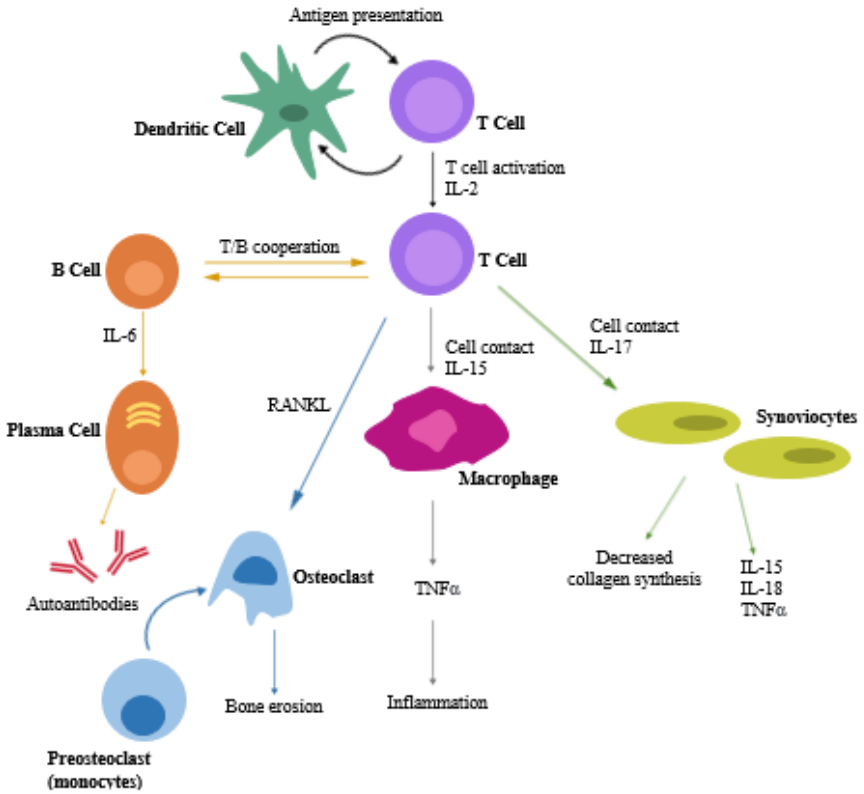

Figure2: *Overview of RA Pathogenesis*

CHAPTER 2
OBJECTIVES

The objectives of the study were to analyze the anti-inflammatory and anti-oxidative potential of the *Thymus serpyllum* seed extract. To create an efficient drug delivery system with high permeability rate. Use of natural compounds with no harmful effects unlike the traditionally used synthetic medications for Rheumatoid Arthritis.

CHAPTER 3
LITERATURE REVIEW

3.1 Role of B-cells in Rheumatoid Arthritis

The innate and adaptive immune system have a relative immunopathogenic role in different stages of RA. The course of RA is described as three stages i.e., disease initiation – it is an antigen independent phase that involves the constituents of joints, perpetuation – it involves the inflammatory phase that is in response to specific antigens, foreign or native, terminal destruction – it comprises of degradation of synovium which is an antigen independent phase. The contribution of B-cells in specific stages of the disease is not yet entirely understood but their role is quite significant in linking of immune cells, associating the innate and adaptive immune systems, and leading inflammation caused by cellular components. Autoantibodies, like RF and ACPA, are used as serological markers for RA and are associated with a severe form of the disease. Enhanced activation of B-cells causes the migration of cells into the synovium via chemokine receptors. This causes the T-cells and B-cells to agglomerate and form tertiary follicular structures which are involved in the expression of CD40 ligands, production of cytokines like IL-6 and IL-10 that further stimulate B-cells, and increased mutational activity in the synovium. The clumping of T-cells and B-cells aggravates the disease, producing higher levels of autoantibodies which are linked with severe symptoms of RA (Burmester, 2003).

3.2 Role of Various T-cell Subpopulations in Rheumatoid Arthritis

In RA, the T-cells activate fibroblasts and macrophages and convert them in to tissue destructive cells. These activates macrophages can also produce a range of cytokines and chemokines that leads to the inflammation of joints. The activation of T-cells by B-cells, macrophages or dendritic cells (DCs) causes the CD4+ T-cells to interact with the HLA and MHC-II molecules. CD4+ T-cells also co-stimulate CD28 molecules expressed on APCs which triggers the initiation of PI3K signaling downstream pathway involved in the

CD4+ cells maturation and antigenic activation of naïve CD8+ T-cells, promoting the inflammatory process. CD4+ T-cells are also associated with the HLA-DR4 allele which leads to an aggressive form of RA. The CD4+ T-helper (Th) cells are also involved in the secretion of cytokines and chemokines involved in the pathogenesis of RA. Type 1 T-helper (Th1) cells secrete IFN-gamma (IFN-γ), TNF- α and IL-2 which are pro-inflammatory cytokines involved in the activation of RA. Another type of CD4+ type 2 T-helper (Th2) cells secrete IL-4 and IL-5 which are the anti-inflammatory cytokines and are involved in the B-cell activation. The Th17 cells secrete IL-17 which triggers the secretion of pro-inflammatory cytokines, chemokine and matrix metalloproteinases (MMPs) and have a high level in the serum of RA patients. IL-17 is associates with the production of VEGF-A, IL-6, IL-8, MMP-1 and MMP-3 in the synovial fibroblasts, and is also involved in osteoclastogenesis, pannus growth and neoangiogenesis.

These multiple T-cells and its associated pathways are involved in the process of chronic inflammation which results in pathogenesis of RA. T-cells also activate B-cells and contribute in the production of antibodies, also leading to the pathogenesis of RA (Yap HY, 2018).

3.3 Role of Cytokines in Rheumatoid Arthritis

Many crucial biological processes like tissue repair, cell growth, inflammation, differentiation, proliferation and regulation of immune response is carried out by various cytokines. They are also involved in the pathogenesis of RA by causing chronic inflammation and joint destruction. This is due to the imbalance between the pro-inflammatory cytokines and the anti-inflammatory cytokines. Pro-inflammatory cytokines TNF-alpha, IL-1, IL-33 and IL-17 etc. are involved in inflammation, cartilage degradation, joint destruction, synovitis and bone erosion. The anti-inflammatory cytokines are the protective immune regulating molecules that subdue the impairment caused by the pro-inflammatory cytokines. IL-4, IL-10, IL-13, IL-27 and IL-35 are the anti-inflammatory cytokines which play a protective role in RA. IL-4 produced by the Th2 cells helps in the bone resorption (Somaiya Mateen, 2016).

3.4 Available Medications for Rheumatoid Arthritis

After the diagnosis of RA, the patient is treated to reduce the disease activity or reach full remission within a period of 6 months to avert joint damage and reduce the long-term disabilities associated with RA. The treatment is generally customized based on the needs of the patients and their health. The factors such as age, overall health, type of joint involved, disease progression and education etc. are kept in mind to establish the treatment regime of that patient. The current drugs available are classified into 3 major categories; non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease-modifying anti-rheumatic drugs (DMARDs).

3.4.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are a large class of drugs with structural and functional diversity. They are weak organic acids with a general structure of an acid moiety attached with an aromatic functional group. The first identified NSAID was salicylates, which was based on the salicylic acid extracted from the bark of willow trees. Aspirin, a commonly used NSAID, is the acetylated derivative of salicylic acid known as the acetylsalicylic acid (ASA). Aryl and heteroaryl acetic acid derivatives are another important class of NSAIDs which include ibuprofen, naproxen and fenoprofen (Samik Bindu, 2020).

NSAIDs work by inhibiting the production of prostaglandins (PGs), a product of arachidonic acid. Arachidonic acid is a cell membrane phospholipid which act as a chemical messenger released from the muscles during tissue damage. The conversion of arachidonic acid into PGs is brought about by the action of cyclooxygenase (COX) enzyme. The two isoforms of the enzyme, COX-1 and COX-2 are involved in regulation of different body functions. COX-1 is associated with synthesis of PG involved in homeostasis and expressed during the basal conditions whereas, COX-2 is expressed during inflammatory and other pathologic conditions. NSAIDs hinder the activity of COX-2 enzyme by inhibiting production of PGs due to inflammation and tissue damage. The inhibition of COX-1 enzyme in different tissues like gastroduodenal mucosa and platelets by NSAIDs cause gastrointestinal bleeding and ulceration which is a common side effect of using these medications (Crofford, 2013).

3.4.2 Glucocorticoids

Glucocorticoids (GCs) are a powerful class of anti-inflammatory and immunomodulatory drugs used in the treatment of rheumatic diseases. The commonly used GCs, which include prednisone, prednisolone, methylprednisolone and dexamethasone, work by affecting primary and secondary immune cells. These drugs restrict the functions of endothelial cells, fibroblasts and leucocytes. They limit the access of leucocytes to the inflammation site and inhibit the production and function of inflammatory humoral factors (F. Buttgereit, 2005). For RA, low doses of GC have been proven to be effective however, the dose can be increased to induce a stronger effect. GCs cause a decrease in the number of inflammatory cell subsets and an increase in the amount of blood leukocytes due to the depletion of adhesion molecules which cause the circulation of neutrophils. The drugs work by inhibiting the helper T cells (TH1) and decreasing the levels of proinflammatory cytokines like interleukins (IL) (IL-1, IL-2, IL-3, IL-6), TNF-α, IFN- γ and IL-17.

GCs not only help to evade the predominant symptoms of arthritis like pain and swelling, but can also prevent joint damage. In addition to improving morning stiffness and fatigue, GCs can cause psychological side effects like anxiety and depression etc. (Joana Fonseca Ferreira, 2016).

3.4.3 Disease Modifying Anti-Rheumatic Drugs (DMARDs)

The traditional DMARDs, namely methotrexate, hydroxychloroquine and sulfasalazine, are reckoned as the foundation of rheumatic treatment. They are able to decrease pain, prevent joint damage, avert loss of function and refine quality of life.

Lately a new class of DMARDs known as biologics have been approved to be used in RA treatment as they have shown to improve symptoms and prevent disease progression. Some important biologics are anti-TNF and anti- IL-1 which are the antagonists of tumor necrosis factor and IL-1 (Carlos G. Grijalva, 2007).

The biologics are proteins genetically engineered from human genes that inhibit specific components in the immune system. In contrast to conventional drugs that alter the whole immune system, biologics target specific immune system components which causes more specific effect and less adverse events (Scott, 2012).

3.5 Treatment Strategies and its Side Effects

Traditionally RA is treated in a pyramid approach. Initially joint pain and inflammation are targeted by the use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs). Later on, as the disease progress, the disease modifying anti-rheumatic drugs (DMARDs) are used as they are more effective but have been proven to be toxic. Studies have shown NSAIDs to be toxic, as they damage the gastroduodenal mucosa leading to increased mortality and morbidity of RA patients. Taking these medicines for more than 1 year increases the chances of stomach ulcers with other minor side-effects like dyspepsia and nausea. NSAIDs can also cause upper GI bleeding and perforated ulcers which causes the relative risk of hospitalization and death by 6.77 and 4.21 respectively.

DMARDs are able to minimize the rate of joint destruction showing a better outcome in relation to joint damage than the NSAIDs alone. DMARDs are also better than NSAIDs in relieving the pain and slowing the progression of the disease related disabilities (J.F.Fries, 2000). Hence nowadays, DMARDs are used as the first-line therapy according to the current clinical guidelines.

Corticosteroids are more potent than NSAIDs but they possess excessive side effects. As a result, they are used in low dosages for a short period of time by either oral medication or by injecting at the site of inflammation. They cause bone thinning, diabetes, weight gain and immunosuppression (Liu D, 2013).

A new treatment strategy is the use of targeted drugs known as the biologics. They provide protection against joint destruction and bone erosion. Depending on the symptoms and stage of the disease, traditional DMARDs and biologics are used in combination to achieve the therapeutic effect with fewer side effects in comparison to using only traditional DMARDs. The biological DMARDs working principal is the blockage of proinflammatory mediators that cause the formation of the characteristics of RA. These include the inhibitors for TNF, inhibitors for CTLA4, molecules targeting the B cell CD20 antigen, IL-6 inhibitors and JAK1 and JAK3 inhibitors etc. These are mostly used in the later stage of the disease when the patients stop responding to the conventional DMARDs (Kritika Chaudhari, 2016).

DRUGS	***MODE OF ACTION***	***ADVERSE EFFECTS***
Non-biologics		
Leflunomide	Inhibits pyrimidine synthesis	Liver effect, skin rash, GI intolerance, teratogenesis
Hydroxychloroquine	Blocks toll like receptors	Rare ocular toxicity, GI intolerance, TB, infections
Methotrexate	Inhibits dihydrofolate reductase	Liver effects, oral ulcers, alopecia, cytopenia, teratogenesis
Sulfasalazine	Folate depletion	GI intolerance, rash, oral ulcers, anemia
Minocycline	Anti-microbial, other mechanisms unknown	Drug induced lupus erythematosus, dizziness
cyclosporine	Calcineurin inhibitor, decreases IL-2	GI intolerance, cytopenia, infections, hypertension, renal disease, hirsutism
Cyclophosphamide	Nitrogen mustard alkylating agent, cross-links DNA	Infertility, cancer, hemorrhagic cystitis
Biologics		
Abatacept	Costimulatory blocker, cytotoxic T lymphocyte antigen 4	Hypersensitivity, infections, ISR, Chronic obstructive pulmonary disease
Adalimumab	Anti TNF-α	ISR, infections, TB, demyelinating disorders
Anakinra	Anti-IL-1 receptor blocker	ISR, leucopenia, infections, hypersensitivity
Etanercept	Anti TNF- α, receptor	ISR, infections, TB, demyelinating disorders

Infliximab	Anti TNF-α	ISR, infection, TB, demyelinating disorders, infusion reaction
Rituximab	Anti-CD20, eliminates B cells	ISR, infection risk, new and reactivation viral infections, respiratory difficulty, cytopenia, progressive multifocal leukoencephalopathy
Tocilizumab	Anti-IL-6 receptor blocker	Infections

ISR: *Injection site reaction* ***TB:*** *Tuberculosis*

Table 2: *List of Non-Biologics and Biologics Drugs with its side effects*

3.6 Microemulsions (ME)

Microemulsions are colloidal drug carrier systems that are transparent and thermodynamically stable. It consists of a mixture of hydrophilic and lipophilic substances which is stabilized by the appropriate ratios of surfactant and co-surfactant due to which it has the capacity to incorporate both hydrophilic and lipophilic compounds in the respective phases. The easy and simple preparation process, enhanced solubility and biocompatibility with long term stability makes it an efficient skin drug delivery system. The stratum corneum is mostly keratin and lipid, the lipophilic phase of microemulsion helps the drug to easily cross this barrier making it the most convenient topical drug delivery system (Tripti Shukla, 2018). Microemulsions also have a high drug loading capacity and it also protects the drug from enzymatic and chemical degradation providing better drug permeability and bioavailability. ME are thermodynamically stable as the phases do not separate overtime and they can stay stable for many years.

ME are formed by the addition of lower alkanols (methanol, ethanol and butanol etc.) in milky emulsions to produce clear and transparent solutions. These solutions can either have dispersions of water-in-oil (O/W) or oil-in-water (O/W) having a size range of around 10nm to 300nm. Due to this small size they appear as optically isotropic and translucent. This particle size can be measured using a dynamic light scattering or neutron scattering technique (Fink, 2020).

The lower alkanols are known as co-surfactants, as they decrease the interfacial tension between the water and oil layers to form a spontaneous micro-heterogenous system. The co-surfactant is mixed with a surfactant like Tween 20, Tween 80 etc. to form an amphiphile which combines the two immiscible liquids like water and oil together. This miscibility of oil, water and amphiphile depends on the composition of the microemulsion system. The compositions of different components can be determined using the ternary and quaternary phase diagrams (Moulik, 2001).

3.6.1 Oil-in-Water (O/W) and Water-in-Oil (W/O) Microemulsions

Microemulsions can be classified into O/W and W/O depending on the composition of the system. The O/W microemulsion has an immiscible oil dispersed in the aqueous buffer with the help of a surfactant and co-surfactant mix. This reduced the surface tension between the oil and water to form stable and equally dispersed droplets. In a W/O microemulsion, water droplets are covered with surfactant and co-surfactant mixture in an oily continuous phase (Jun Cao, 2008).

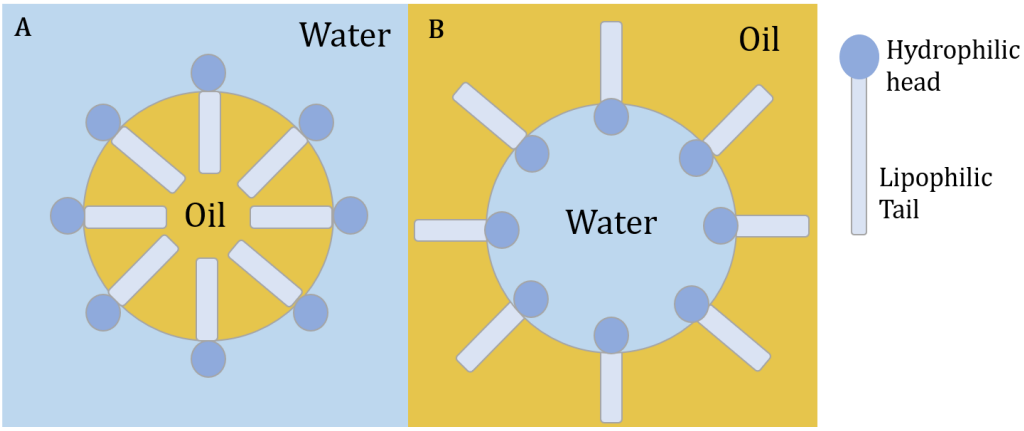

Figure 3: A. *Oil in water (O/W) Microemulsion* ***B.*** *Water in oil (W/O) Microemulsion*

3.6.2 Topical microemulsions for Rheumatoid Arthritis

The effectiveness of topical drug administration depends upon the composition and the phase behavior of the microemulsion. As we know that the co-surfactant reduces the

interfacial tension between the two immiscible phases resulting in a decreased droplet size and an increased drug release rate. The surfactant, oil and alcohol components of microemulsions can alter the structural integrity of stratum corneum to provide a passage for the movement of drug across the skin barrier (SylwiaKupper, 2017). For the efficient transdermal and topical delivery of drugs the components of microemulsion should be biocompatible, non-toxic and should have a low skin irritability.

3.7 Ethnobotanical Medicines for Treatment of Rheumatoid Arthritis

Since the early times, herbal medicines have been used for different diseases and this information has been passed on from generations to generations. Due to its safety and cost effectiveness, medicinal plants have gained quite an interest in the pharmacological industry. Use of plants in ethnobotanical medicines comes from years of experience and can be as old as the man himself. Not all parts of the plant are used as there is a difference in the distribution of phytochemicals used against the disease in the different parts of plant. Literature shows the use of medicinal plant for the treatment of RA. The synthetic medicines used in the management of RA have severe side effects and are expensive due to its overlong uptake. As a resultant, there is a paradigm shift towards the ethnomedicines which have proven to be safe and effective. The scientific information available on these plants is very little and its medicinal properties are required to be scientifically validated. In Jos Plateau State of Nigeria, ginger and garlic are the predominantly used plants for treating RA. Not all parts of the plant are used as there is a difference in the distribution of phytochemicals used against the disease in the different parts of plant. Bulb and stem are the most abundant parts of these plants that are used (Tongshuwar, 2020).

In Pakistan there are about 6000 different wild species of plants out of which 400-600 species are considered to be therapeutically important. About 84% of Pakistanis use herbal remedies for their ailments. 371 medicinal plant species belonging to 263 genera and 99 families are being used as traditional medication of inflammatory disorders in Pakistan. Majority of these plants belong from the Punjab and Khyber Pakhtunkhwa regions. A huge

number of these plants belonging to the Asteraceae and Fabaceae family are shrubs and herbs. Leaves are the most commonly used plant part for ethnomedicine used in the form of decoctions for oral consumptions. *Berberis lycium Royle, Citrullus colocynthis (L.) Schrad, Calotropis procera* (Wild.), *Datura innoxia Mill, Ricinus communis L, Solanum nigrum L, Solanum Surattense Burm. F* and *Verbascum thapsus L.* are the most commonly used medicinal plant in the treatment of inflammatory diseases in Pakistan (Alamgeer, 2018).

3.8 *Thymus serpyllum*

Thymus serpyllum also commonly known as the wild thyme or Breckland thyme is a native perennial shrub of northern and central Europe. The name "*serpyllum*" comes from a Greek word meaning "to creep" due to the crawling movement of the thyme. It has a long woody stem with oval leaves arranged in a rosette formation at the top. The inflorescences are tall and in series along the low-lying stems. The top of the stem has a verticillaster arrangement of flowers that grows from May to September. *Thymus serpyllum* grows best in stony or dry grounds and even grasslands.

The use of wild thyme for the purpose of traditional medicine has been a centuries old practice. The upper part of the plant contains essentials oils with certain healing properties

Figure 4: *Thymus Serpyllum L.*

that can be used as fresh or dry herbs. *Thymus serpyllum* is used as an herbal medicine in

the form of tinctures, decoctions, syrups, infusions, oil and tea. This has led to its use in the chemical and pharmaceutical industries. Now a days, consumers are looking at natural and safe products for their clinical applications as compared to the synthetic chemicals, which are generally considered as toxic for having several side effects. This has caused a major interest in the phytochemical properties of traditionally used medicinal and aromatic plants like *Thymus serpyllum* (Snežana Jarić, 2015).

3.8.1 Taxonomic Hierarchy of *Thymus serpyllum*

In Pakistan, *Thymus serpyllum* is commonly known as "Ajwain Jungli". The taxonomical classification of *T. serpyllum* is as follows:

Scientific Classification	
Kingdom:	Plantae
Clade*:*	Tracheophytes
Clade*:*	Angiosperms
Clade*:*	Eudicots
Clade*:*	Asterids
Order:	Lamiales
Family:	Lamiaceae
Genus:	*Thymus*
Species:	***T. serpyllum***

Table 3: *Scientific Classification of Thymus serpyllum*

3.8.2Ethnopharmacology of *Thymus serpyllum*

Traditional use of the genus *Thymus* stems from ancient Egypt, where it was used to make fragrant balms for medicinal purposes. According to Dioscorides, it was also used to treat upper respiratory tract infections and for stomach ailments (Heilmeyer). Thyme is thought to be brought into the Europe by the Romans who used it to enhance the flavor of their foods and drinks. Europeans used the herb underneath their pillows to aid sleep (Huxley, 1992). According to the Chilandar Medical Codex (15^{th}- 16^{th} century), wild thyme was

used to treat diseases of respiratory tracts and digestive problems (S. Jarić, 2014). In the Renaissance period it was used for the treatment of malaria and epilepsy (M. Adams, 2012). The upper parts of thyme are used as an antiseptic, anthelmintic, disinfectant, antispasmodic, sedative and expectorant. *T. serpyllum* is commonly used against disorders concerning gastrointestinal and respiratory system.

3.8.3 Pharmacological Properties of *Thymus serpyllum*

Essential oil of *Thymus serpyllum* show significant antibacterial activity against the cariogenic *Streptococcus mutans* species. The oil was also effective against Candida spp. Like C. krusei, C. tropicalis and C. glabrata which are involved in the cause of human cavities. The literature shows that *T. serpyllum* oil can be used against human pathogens that cause oral diseases. This activity can be attributed to thymol which is the major constituent of thyme oil.

Several studies have reported the antioxidant potential of *T. serpyllum* oil which proves its traditional use as a food additive. This antioxidant potential is considered to be due to the phenolic constituents of the oil. Antioxidant property of thyme oil makes it a potential chemoprotective against oxidative stress mediated diseases which is due to its radical scavenging and metal chelating properties.

T. serpyllum oil has also shown to have antitumor properties. The oil is most effective against PC3, A549 and MCF-7 cell lines. Carvacrol is the major oil constituent that shows *in vitro* cytotoxic activity against the cancer cell lines. Thyme oil shows a concentration dependent viability of cell lines (Miloš Nikolić, 2014).

CHAPTER 4
METHODOLOGY

4.1 Plant Extract Preparation

Thymus serpyllum seeds were ordered from an online website (pansari.pk). The seeds were crushed using a mechanical grinder and sieved to obtain a fine powder. The Soxhlet apparatus was used for the extraction procedure. 150g of powdered seeds was placed in the thimble chamber of the Soxhlet apparatus. Pure ethanol was used as the extracting solvent. 200ml of the solvent was placed in the round bottom flask. The temperature was kept at around 50^oC - 60^oC which is lower than the boiling point of ethanol. The apparatus was kept running until transparent solvent is seen through the siphon arm. The liquid was filtered and dried at 37^oC to obtain dry plant extract (Mahire, 2020).

4.2. Phytochemical Analysis of Thymus serpyllum Seed Extract

The presence of phytochemical components was screened in plant extract using the standard protocols.

4.2.1 Qualitative Phytochemical Analysis

4.2.1.1. Saponins

Saponins possess anti-inflammatory and anti-septic properties. 0.5ml of plant extract was mixed with 5 ml of hot distilled water. The solution was shaken vigorously for the formation of 1 cm layer of froth over the surface.

4.2.1.2 Anthraquinones

Anthraquinones play role in the anti-inflammatory and anti-microbial activity of the plant extract. 3ml of plant extract was mixed with 3 ml of benzene. 5ml of 10% ammonia was added to give a pink to red color formation.

4.2.1.3 Flavonoids

In addition to anti-oxidant activities, flavonoids also have anti-cancerous and anti-viral properties. 1ml of plant extract was mixed with 1ml of 10% lead acetate to give yellow precipitates.

4.2.1.4. Phenols

Phenols help in slowing down the progression of degenerative diseases as it possesses anti-inflammatory and anti-oxidant activities. 1 ml $FeCl_3$ (1%) was added to 1ml of plant extract to give a blueish- greenish color.

4.2.1.5 Alkaloids

Alkaloids show anti-inflammatory, anti-cancerous and analgesic properties in humans. Hager's test is used for the identification of alkaloids. 2 ml of plant extract are treated with a few drops of Hager's reagent. Presence of yellow precipitates indicate the presence of alkaloids.

4.2.1.6 Amino acids

Amino acids are the building blocks of proteins and are considered vital for proteins, hormones and neurotransmitters. Ninhydrin test was used to check the presence of amino acids. In 1 ml of extract a few drops of ninhydrin reagent was added. The mixture was heated in a water bath at boiling temperature. A blue purple coloration indicates the presence of amino acids.

4.2.1.7 Carbohydrates

For screening the presence of carbohydrates, Fehling's test was used. Fehling's solution A and B were prepared ahead of performing the test. In 2 ml of plant extract, equal volumes of Fehling's solution A and B were added. The mixture was boiled for a few minutes and the presence of brick red precipitates indicates the presence of reducing sugars.

4.2.1.8 Cardiac Glycosides

Cardiac glycosides can be used to develop drugs for congestive heart failure as these are a type of steroids that stimulate the cardiac muscles. Small dose cardiac glycosides can be used for different heart disease (Morsy, 2017). 2 ml of plant extract was treated with 500 ul of glacial acetic acid and a few drops ferric chloride. After mixing, 1 ml of Sulfuric acid was added by pipetting along the edge of test tube. The presence was confirmed by the appearance of reddish-brown interference at the junction of the two liquids.

4.2.1.9 Steroids

1 ml of plant extract was added in 2 ml of chloroform. 1 ml of concentrated sulfuric acid was added by pipetting along the sides of the test tube. The upper layer changes to reddish color indicating the presence of steroids.

4.2.2. Quantitative Phytochemical Analysis

4.2.2.1 Determination of Total phenolics content (TPC)

This method is based on the transfer of electrons in the alkaline medium by the phenolic compounds present in the sample. This forms a blue chromophore of tungsten and molybdenum. The maximum absorption is related to the concentration of phenolic compounds present.

Folin Ciocalteu phenol (FC) reagent was used for the analysis. The concentrations of sample were made in distilled water. 0.5ml of sample was mixed with 2.5ml of freshly prepared FC reagent (10%). After 5 minutes, 2ml of 7.5% sodium carbonate solution was added. The mixture was incubated at 40^{o}C for 1 hour before measuring the absorbance at 725 nm. Gallic acid was used as a standard and the amount was calculated as mg gallic acid equivalents per gram of sample (mg/g).

4.2.2.2 Determination of Total Flavonoids Content

The sample concentrations were prepared in methanol. In 4ml of distilled water, 100ul of sample was added. Then, 300ul of 5% of sodium nitrate solution was added. After 5 min, 300ul of aluminum chloride (10%) was added. After another 6 min, 2ml of sodium

hydroxide solution (1M) was added with an immediate addition of 3.3ml of distilled water. the mixture was vortexed and the absorbance was measured at 492nm. Rutin was used to calibrate the standard curve and the calculated values were expressed as mg Rutin equivalents per gram of sample (mg/g) (Sadegh Fattahi, 2014).

4.3 In-vitro Anti-oxidant Assays

4.3.1. DPPH (1, 1-diphenyl- 2-picrylhydrazyl) Assay

It is the most commonly used in-vitro anti-oxidant assay for plant extract. The assay is based on the free radical method. The hydrogen donating capability of plant extract is determined by the decolorization of violet/purple methanol solution of DPPH to a yellow color.

0.1mM methanolic solution of DPPH is prepared 1 hour prior to the analysis. The solution is kept in dark as it is light sensitive. Different concentration of methanolic solutions of sample were prepared. Ascorbic acid was used as a standard for this assay. 50ul of the test sample at various concentrations was added in the 96 wells plate. 150ul of DPPH solution was added to the plate and was incubated for an hour in the dark (Farahziela Abu, 2017). The absorbance was calculated at 550nm and the activity was expresses as Ascorbic acid equivalent per gram dry weight (mg AA/g dry weight) and was calculated using the formula:

$$\text{DPPH radical scavenging activity} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100.$$

4.3.2 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay determines the antioxidant potential of plant extract by the change of color due to the conversion of ferric (Fe+3) ion to a ferrous (Fe+2) ion. This is done by the donation of a hydrogen by the plant extract to break the free radical chain.

Different concentrations of sample were prepared in methanol. Gallic acid was used as a standard. 1 ml of sample was diluted in 5ml of water in a tube. 1.5 ml of 1M HCL, 1.5ml of potassium ferricyanide (1%), 0.5ml of SDS solution (10%) and 0.5ml of ferric chloride solution (0.2%) was added to the tubes. The tubes were vortexed and incubated at 50^{o}C for

20 mins. Absorbance was calculated at 750nm using a spectrophotometer. The values were expressed as milligram gallic acid equivalent per gram of dry weight (mg GAE/g dry weight) (Ruckmani, 2016).

4.4 In-vitro Anti-Inflammatory Assays

4.4.1 Albumin Denaturation Inhibition Assay

The assay works on the principal that the proteins can lose its tertiary and secondary structures by the application of external stress like heat and compounds.

A dilution series of methanolic extract and 1 % aqueous solution of ovalbumin was prepared. Phosphate buffer saline (PBS) of 6.4 pH was also prepared. The reaction mixture consisted of 200ul of ovalbumin, 2.8ml PBS and 2 ml of various concentrations of sample. This mixture was incubated at 37^{o}C for 15 mins, after the temperature was increased to 70^{o}C for 5 mins. The mixture was allowed to cool before measuring its absorbance at 450nm. Diclofenac sodium was used as a standard.

The formula used was:

$$\% \text{ inhibition} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100.$$

4.4.2 Membrane Stabilization Assay

Fresh whole human blood was centrifuged at 3000 rpm for 10 mins at 4^{o}C and was washed three to four times with 0.9% saline solution. The blood pellet formed at the end of used to prepare a 10% blood suspension in PBS (6.4 pH). The inhibition of membrane stabilization was calculated by using different concentrations samples. 500ul of the sample was added in 1ml of normal saline (0.9%).500ul of blood suspension and 1ml of PBS was added. The tubes were vortexed and incubated at 37^{o}C for 30 min. The tubes were then centrifuged at 2500 rpm for 5 mins. Absorbance was calculated at 550nm and the reading was taken in triplicates. The formula used was:

$$\text{Membrane Stability} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100.$$

4.5 Toxicology Assays

To analyze the toxic properties of the *Thymus serpyllum* seed extract before in-vivo testing following assays were conducted.

4.5.1 In-vitro Hemolytic Assay

In hemolytic assay, extracellular environment of early endosomes and late endo-lysosomes is mimicked by incubating red blood cells and test materials with solutions of defined pH. The amount of hemoglobin released into the solution is measured using a spectrophotometer. The percent of red blood cells disrupted are than quantified by comparing with the positive and negative controls. The erythrocyte membrane is used as a substitute to the lipid bilayer membrane of the lysosomes.

The protocol was conducted with a few modifications (Evans BC, 2013). Whole human blood in EDTA tubes is centrifuged at 500 x g for 5 minutes at 4^{o}C. Remove the plasma layer and replace it with 9% saline solution for washing. Repeat the washing step 3 to 4 times for packing red blood cells. Make 2% erythrocyte solution by adding the packed red blood cells in PBS (7.4 pH). Serial dilutions of plant extract in PBS are made. Add 30ul of plant sample with various concentrations and 550ul of blood suspension in a tube and incubate at 37^{o}C for 1 hour. 10% triton-X is used as a positive control and PBS is used as a negative control. After incubation the tubes are centrifuged at 500 x g for 5 mins to form blood pellets. The absorbance of the mixture is measured at 405nm and the readings are compared with controls. The formula used is as follow:

$$\% \text{ Hemolysis} = \frac{\text{Abs sample} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100$$

4.5.2 In-vivo acute oral toxicity (LD50) study

In this study, female BALB/c mice were used to observe the toxic effect of crude ethanolic extract of *T. serpyllum* seeds. Throughout the study, mice were observed for adverse toxicity signs and mortalities. Physical appearance of the mice was also considered as it indicates the toxic effect of extract (Abdu Zakari, 2018).

Approval of experiments on mice model was acquired from institutional review board committee with Performa number 32-IRB-ASAB-2016 at Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Pakistan.

The parameters considered during the study were body weight, paw licking, calmness, erect fur, sleep and death.

The assay was conducted in three phases. The mice were color coded and weighed to separate into three randomized groups for each dose. In phase I, 20ml of three doses of 30mg/kg, 300mg/kg and 500mg/kg were used. In phase II, 1000mg/kg, 2000mg/kg and 3000mg/kg of doses were administered. In phase III, 5000mg/kg of dose of plant extract was used, which is the limit dose as provided by the EPA/OECD and considered as high enough dose to test for acute toxicity. The doses were prepared using the formula:

$$\text{Extract volume} = \frac{\text{weight of mice} \times \text{Dose}}{\text{Stock concentration}}$$

Here the dose refers to the expected therapeutic level of the plant.

4.6 Microemulsion Formulation

4.6.1 Screening of Components for Microemulsion

The saturated solubility of *T. serpyllum* seed extract was measured in different oils and surfactants like castor oil, oleic acid, ethanol, tween 20 and tween 80. For solubility testing, excess of crude plant extract was added to fixed volumes of oils and surfactants. The extract was mixed by vortex for 5 minutes and sonication for 1 minute. The mixture was then kept at 37^{o}C in a shaking water bath for 24 hours. The mixture was then centrifuged and excess drug was allowed to settle at the bottom. The contents were filtered and the solubility was determined spectrophotometrically at 405nm after appropriate dilutions.

4.6.2 Construction of Pseudo-Ternary Phase Diagrams

To determine the microemulsion regions, phase diagrams of oil, surfactant/co-surfactant (Smix) and water were constructed using the titration method. Smix of different weight ratios (Km) both in the increasing and decreasing order of co-surfactant related to surfactant were used. Different weight ratios of oil and Smix (1:9, 2:8, 3:7, 4:6, 5:5, 6:4,

7:3, 8:2, 9:1) were slowly titrated with the aqueous phase at 37^{o}C under constant shaking. After being equilibrated, the mixtures were visually assessed as microemulsion due to its clarity and transparency. The point at which the mixtures become turbid was marked on the pseudo-ternary phase diagram with water, oil and Smix representing the three axes. Chemix school software was used to construct the phase diagrams.

4.6.3 Formulation Development

From the Microemulsion regions identified in the pseudo-ternary phase diagrams, different formulations were picked from each phase diagram which was further screened for thermodynamic stability. To make microemulsions with various concentrations of oil, water and Smix, first the volume of oil is mixed with the Smix and mixed. The appropriate amount of lukewarm distilled water is added in a drop wise manner to get a clear and transparent mixture.

4.6.4 Formulation Stability Testing

The thermodynamic stability of prepared microemulsions was evaluated using different stability tests. Following tests were performed

4.6.4.1 Centrifugation

Selected formulations were centrifuged at 3500 rpm for 30 minutes. The formulations were evaluated for phase separation.

4.6.4.2 Heating Cooling Cycle

The formulations were stored at six cycles between 4^{o}C and 45^{o}C with storage at each temperature of not less than 48 hours were done. The formulations were checked for phase separation.

4.6.4.3 Dilution Test

The microemulsions formed were diluted in 1:10, and 1:100, ratios with double distilled water to check if the system shows any signs of separation.

4.6.4.4 Freeze Thaw Cycle

The formulations were stored at -21ºC and 25ºC for three cycles for less than 48 hours. The formulations were checked for stability by evaluating the phase separation.
The formulations that survived the stability tests were selected for further study.

4.6.5 Calculation of Percentage Transmittance

The percentage transmittance of the seven formulated microemulsions was also calculated to determine the transparency of the microemulsions. The absorbance of microemulsions was calculated at 550nm. The formula used to calculate the value was:

$$\% \text{ Transmittance} = \text{Antilog } (2 - \text{Abs})$$

Where Abs is the absorbance of the microemulsion.

4.6.6 Identification of The Type of Microemulsion

The final prepared microemulsion looks transparent and clear, hence there is no way to distinguish between the O/W and W/O microemulsion. A few tests are able to help conclude the type of microemulsion formed.

4.6.6.1 Dye Solubility Test

For this test water soluble and oil soluble dyes are used. When a water-soluble dye is added to the O/W microemulsion it will appear in the continuous phase that is water, as it will dissolve itself in it. For an oil soluble dye for O/W microemulsion, the continuous phase would appear colorless as the dye is unable to dissolve in it and the scattered globules of oil will be colored.

4.6.6.2 Formation of Creaming

The sample is heated in a test tube for 10 minutes and observed. For an O/W microemulsion, upward creaming can be observed. Whereas for a W/O microemulsion, downward creaming can be observed.

4.6.6.3 Cobalt Chloride Test

A filter paper soaked in cobalt chloride is dried and dipped in the microemulsion to identify the type of microemulsion present. If the whole filter paper turns pink it indicates the presence of O/W microemulsion as anhydrous cobalt chloride turns pink as it absorbs water. In case of W/O microemulsion, pink spots will appear on the filter paper.

4.6.7 Preparation of Microemulsions Embedded with *T. Serpyllum* Seed Extract

The formulations that passed the stability tests were chosen for the preparation of *T. serpyllum* extract based microemulsions. Specific amount of extract is mixed by sonication into the required ratio of oil and Smix. After it is fully dissolved the appropriate amount of water is added to make a clear and transparent oil-in-water microemulsion.

4.6.8 Formulation Characterization

The prepared microemulsions were characterized for pH, droplet size, PDI, zeta potential and viscosity. The pH was measured using a pH meter and pH strips. Size and zeta potential were measured using a Zetasizer. The viscosity of the formulations was measured using a viscometer.

4.7 In-vivo Skin Irritation Study

The in-vivo skin irritation study was conducted on female BALB/c mice issued from the ASAB animal house. Ethical approval was acquired from institutional review board committee with Performa number 32-IRB-ASAB-2016 at Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Pakistan.

Draize patch test method was used in which mice were shaved to expose its skin from back. One mouse was applied 1g of a commercially available skin ointment and 1g of empty microemulsion was applied on the back another. The area where the formulation and ointment were applied, was covered with cotton and tape for 4 hours. After which the area was cleaned and mice were assessed for the next 72 hours for erythema and edema.

The microemulsion was given an irritation score on the basis of observations. The observations include signs of itching or changes in skin color, erythema, dryness or flakiness, papule formation and edema.

4.8 In-Vivo Anti-Arthritic Activity of Prepared Microemulsion

To validate the anti-arthritic activity of the prepared microemulsion, 10 male and 10 female BALB/c mice were used. The mice were kept at ASAB animal house in laboratory conditions of 25ºC and 55% humidity. Mice were weighed and acclimatized for a week with regular feed and distilled water.

All experimental procedures to be followed in the study were permitted from IRB at ASAB, National University of Science and Technology (NUST). Healthy mice were chosen, they were screened for the pathogenic anomaly. All mice were kept in laboratory cages. Experimentation was done to meet the criteria of study according to guidelines of the National Institute of Health for using animals in research and experimentation.

4.8.1 Arthritic Mice Model Preparation

Collagen-induced arthritic (CIA) models were prepared by injecting the mice with Bovine Collagenase Type 2 and Freud's Complete adjuvant (FCA). The induced arthritis gave mice symptoms that were similar to Rheumatoid Arthritis in humans.

The emulsion was prepared in 0.1M glacial acetic acid. 0.1M glacial acetic acid was prepared in Hartmann solution. The emulsion was prepared on ice to maintain it from separating. The bovine collagenase was solubilized in glacial acetic acid in 2:1 one day before the induction. 1ml of this solution was mixed with 1ml of FCA. FCA was slowly added to the solution and mixed. The mixture was mixed thoroughly until it was pure white in color by using vortex for 2-3 minutes. 200ul – 100ul of this emulsion was filled in 1ml insulin syringes which were kept on ice to prevent phase separation.

All mice were tagged, weighed and measurements of left hind paw was taken with the help of a Vernier caliper. The first injection (200ul) was given in the tail carefully to prevent puncturing the vein. The tail became white, insuring correct administration of the injection. This day was recorded as day 0. The next injections (100ul) were administered in the left hind paw on day7 and day 14.

Weight and paw measurements were taken on these days as well.

4.8.2 CIA Scoring of Mice Models

The mice were scored for CIA on a scale of 0-4 by visualizing its left hind paws. The mice with a score of 3-4 were used for treatment studies. The scoring was as follows:

PAW SCORE	***OBSERVATIONS***
0	Normal paw. No obvious difference with the healthy mice paw
1	One or two swollen/inflamed toes with normal paw and ankle
2	Three or more toes effected but no paw swelling Or Mild paw swelling
3	Swelling of entire paw
4	Severe swelling of entire paw and toes Or Ankylosed paw and toes

Table 4: *CIA Scoring*

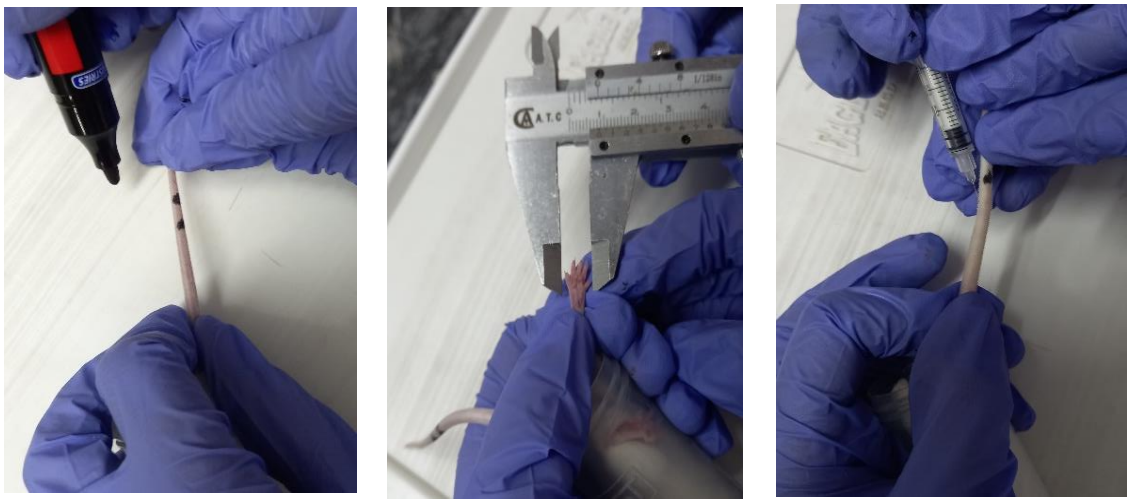

Figure 5: *Tagging, Measuring Paw and Injecting Collagenase and FCA Mixture*

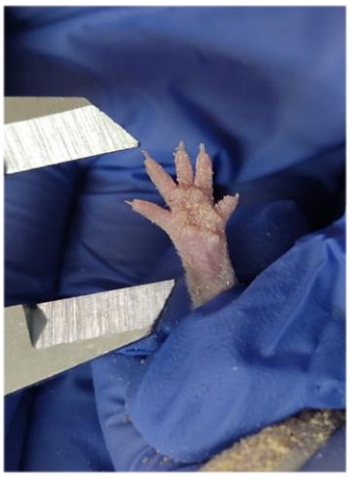

Figure 6: *Normal Paw*

Figure 7: *Arthritic Paw*

4.8.2 Grouping of Mice Models

Both male and female mice were divided into 5 groups with two mice each. The grouping was done as follows:

GROUP NO.	*GROUP NAME*	*CHARACTERISTICS*
1.	Treatment 1	Arthritic mice treated with empty microemulsion
2.	Treatment 2	Arthritic mice treated with *Thymus serpyllum* extract embedded microemulsion
3.	Negative Control	Arthritic mice given no treatment
4.	Positive Control	Arthritic mice given commercial RA medication (Leflunomide)
5.	Normal	Healthy mice

Table 5: *Grouping of Mice*

4.8.3 Administration of Thymus serpyllum Embedded Microemulsion and Standard Drug to Mice Models

Group 1 named treatment 1 and was given microemulsion without the *Thymus serpyllum* extract. Group 2 named treatment 2 was given microemulsion containing 0.8% of *Thymus serpyllum* extract. Group 4 was the positive control and was given Leflunomide, which is

a commercialized DMARD for RA, by mixing 10mg/kg of the drug in distilled water. The Treatment was started two weeks after the induction of arthritis and was continued for 14 days.

4.8.4 Paw Size Measurements

Size of left hind paws was measured with a Vernier caliper of 0.05mm least count. The measurements were taken before injections for inductions, before the beginning of the treatment and after the treatment completion. The measurements were recorded in cm.

4.8.4 Collection of Blood, Liver, Spleen and Paw

On day 46, mice were anesthetized with chloroform. Blood was collecting by cardiac puncture for clinical parameter like RF. Paw samples were collected for X-rays and histopathology. Spleen was collected for performing spleen indexing. The results of all five groups were compared to assess the therapeutic potential of the prepared microemulsion formulations.

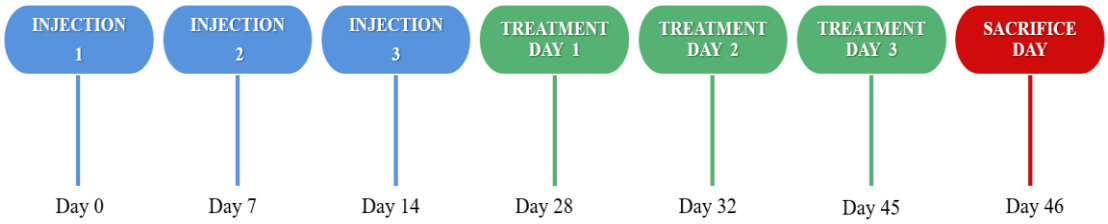

Figure 8: *Schematic Diagram of Arthritis Induction and Treatment*

4.8.5 Histopathology of Ankle Joints and Liver

The left hind paw of mice models was collected for histopathology. It was done to see the difference between the tissue morphology of different groups and to study the effect of formulated microemulsion on RA pathogenesis.

After the sacrifice of mice, the samples were collected in 10% neutral buffer formalin and stored at 4^{o}C for preservation. For paw histopathology the samples were decalcified with 4% nitric acid and kept overnight for better sectioning of the tissues. For fixation, the samples were submerged in 70%, 80% and 100% isopropanol for 2 hours each. After this, the samples were kept in ratios of 5:5 and 3:7 of isopropanol: xylene for 2 hours. Xylene

was used to wash out isopropanol alcohol. After fixation step, the samples were embedded with paraffin into the cassettes. This provides support for fine and thin slicing of tissue samples. The cassettes were refrigerated for 5-10 minutes for solidification of paraffin. After refrigeration, the paraffin block was sliced into thin films of 20um by the use of a microtome. The paraffin slices had thin layers of sample tissues within it. The films were placed over warm water bath and a slide was used to pick the films from underneath. The slides were placed on a hot plate to remove excess paraffin wax exposing the tissue sample. The samples were stained with hematoxylin and eosin stains for better visualization and contrast between different tissues. Cover slips were placed on top of the stained sample for protection and visibility under a microscope.

4.8.6 Spleen Indices

The spleen samples were taken for the purpose of comparing the spleen enlargement between the groups. It was calculated using the formula:

$$\text{Spleen indices} = \frac{\text{Weight of thymus or spleen (mg)}}{\text{Bodyweight (g)}}$$

4.8.7 Biochemical Assays

Blood samples were taken from all groups by cardiac puncture method using 1ml insulin syringes. The harvested blood was immediately transferred to anticoagulation blood collection tubes. Serum was collected and used to check the serum levels of RF in different groups.

4.9 Statistical Analysis

The results are represented as mean ± standard deviation. Statistical analysis was performed with the use of GraphPad prism software. Two-way ANOVA and multiple t-test was conducted to calculate the significance of the obtained values. $P \leq 0.05$ is considered as an indication of statistically significant difference values.

CHAPTER 5
RESULTS

5.1 Thymus serpyllum Extract Preparation

The extract was made with absolute ethanol. As it was a seed extract, the color was light brown and had an earthy scent. After the extraction procedure, the extract was poured into petri dish and left for air drying. The dry extract was sticky and stored at 4^{o}C in a covered tube.

5.2 Phytochemical Analysis of Plant Extract

5.2.1 Qualitative Analysis

The qualitative analysis was performed on ethanolic extract. The results were based on the physical changes observed. The list of the results is summarized as:

TEST NAME	***RESULT***	***Observation***
Phenols	+	Blueish green color
Flavonoids	+	Intense yellow color
Anthraquinones	+	Red color
Saponins	+	1 cm thick layer of foam
Alkaloid	-	No Yellow color ppt
Amino acid	-	No purple coloration
carbohydrate	-	No violet color
Cardiac glycoside	+	Reddish-brown interface
Steroid	+	Upper layer turned red

Table 6: *Results of phytochemical screening of plant extract*

Figure 9: *Negative Results for Amino Acids in Plant Extract*

Figure 10: *Positive Results of Phenols in Plant Extract*

Figure 11*: Negative Results for Alkaloids in Plant Extract*

Figure 12:*Positive Results for Flavonoids in Plant Extract*

Figure 13: *Positive Results of Anthraquinones in Plant Extract*

Figure 14:*Positive Result of Saponins in Plant Extract*

Figure 15: *Positive Results for Steroids in Plant Extract*

Figure 16: *Positive Results for Cardiac glycosides in Plant Extract*

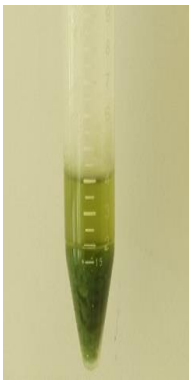

Figure 17: *Negative Results for Carbohydrates in Plant Extract*

5.2.2 Qualitative Analysis

For qualitative analysis of phytochemicals, total phenolics and total flavonoids content was calculated.

5.2.2.1 Total Phenolics Content

The value for total phenolic content was measured using the Folin-Ciocalteu reagent in terms of gallic acid equivalent. The total phenolic concentration is represented as mg gallic acid equivalent of per gram weight of dry extract. The standard graph of gallic acid was prepared by using linear regression. The value was calculated using the linear regression equation **(y=0.0001x+0.0562)** by putting the value of plant extract absorbance at "y" and calculating the value of "x". The concentration was then calculated by using the formula:

$$C = x \cdot V/m \quad \text{---------- (equ. 1)}$$

Where "V" is the volume of extract used and "m" is the mass of the plant extract. The calculated values of total phenolic content in Thymus serpyllum seed extract was **94 ± 0.0048 mg GAE/g of dry extract weight.**

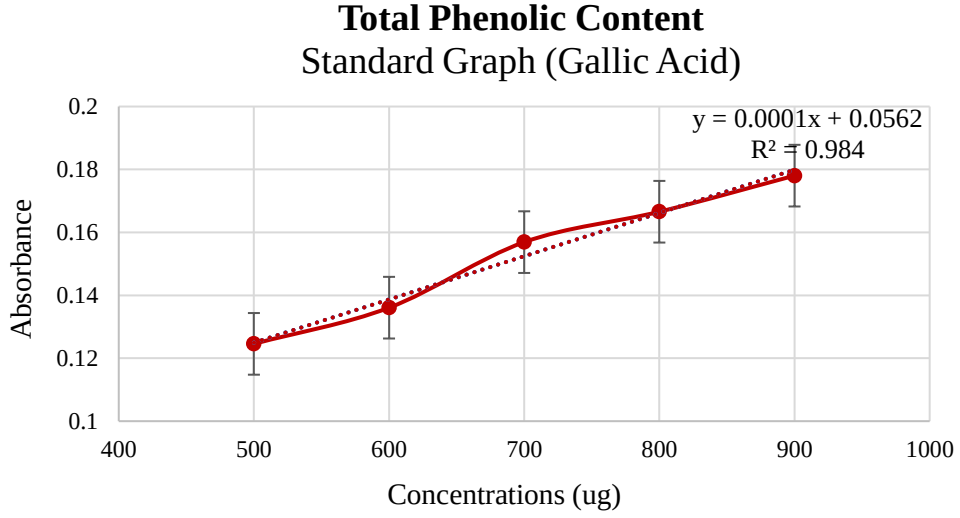

***Figure 18**: Graph for Total Phenolics Content*

5.2.2.2 Total Flavonoids Content

The total flavonoid content for ethanolic seed extract of Thymus serpyllum was measured using the aluminum chloride colorimetric assay. Rutin was used to create a standard curve. The results are expresses as mg Rutin equivalent per gram weight of dry extract. The graph results were obtained by linear regression and the value for total flavonoid content was calculated using the linear regression equation **(y=0.0001x + 0.02)**. By putting the value of absorbance of plant extract in the equation and using the equation 1 the value for total flavonoids content was **828 ± 0.009 mg RE/g of dry weight extract.**

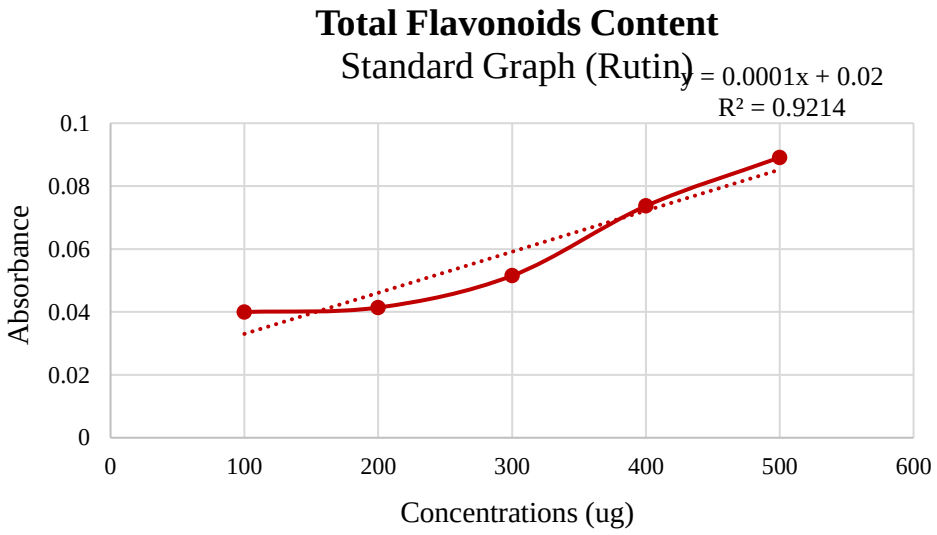

Figure 19: *Graph for Total Flavonoids Content*

5.3 In-Vitro Anti-Oxidant Assays

5.3.1 DPPH Assay

The results of DPPH assay were assessed by measuring the percentage inhibition and radical DPPH scavenging activity of *Thymus serpyllum* extract was expressed as mg Ascorbic Acid equivalent per gram dry weight of plant extract. Ascorbic acid was used as a standard. Standard graph was calculated using the linear regression. The linear regression equation was used to calculate the DPPH scavenging activity and was found to be **176.901 ± 0.001 mg AAE/g of dry weight plant extract.**

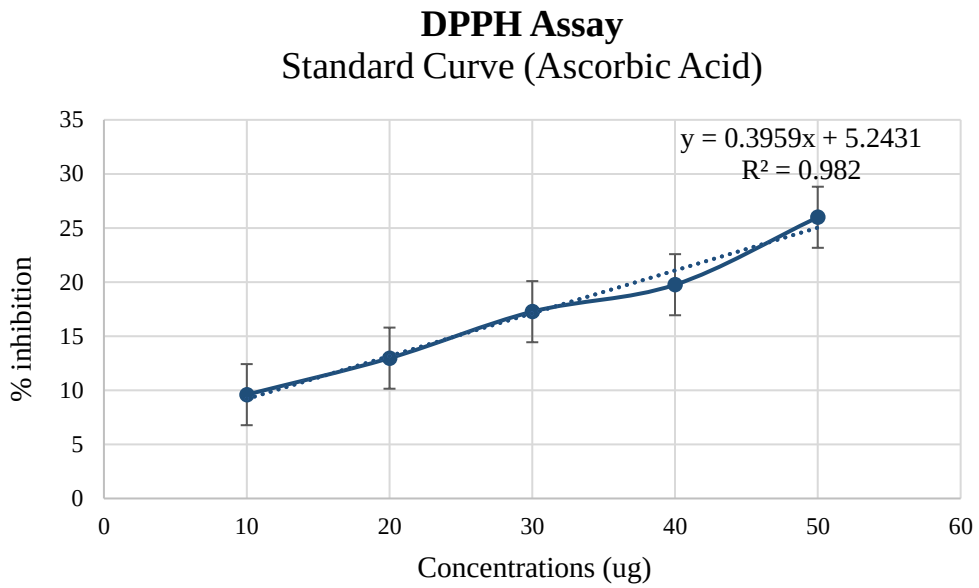

Figure 20: *Standard Curve for DPPH Assay*

5.3.2 FRAP Assay

For this assay, gallic acid was used to calibrate the standard curve. The absorbance was measured by a spectrophotometer at the wavelength of 750nm. The linear regression curve obtained from the gallic acid standard curve was used to calculate the value of FRAP as

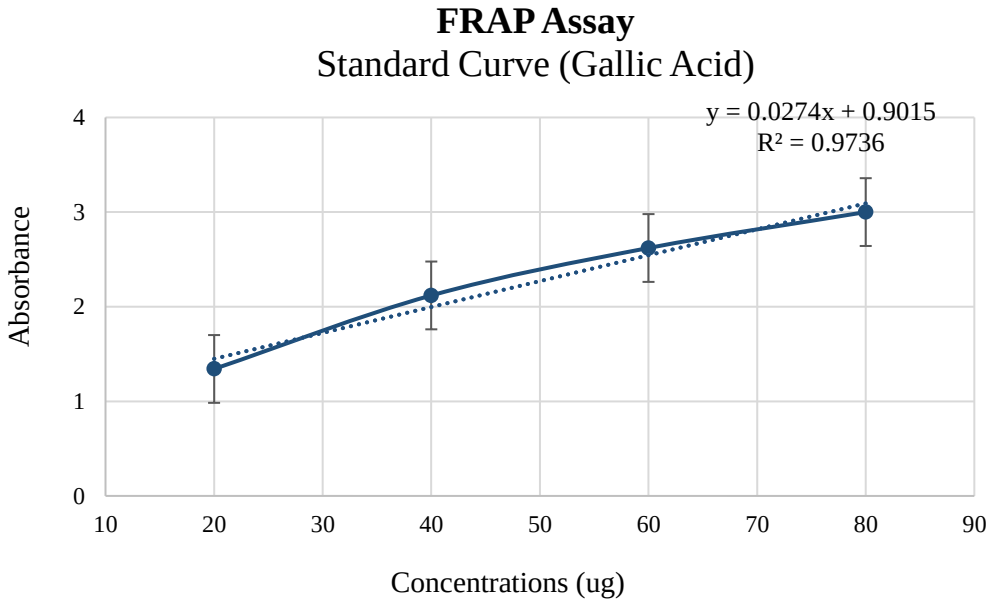

Figure 21: *Graph of FRAP Assay*

mg gallic acid equivalent per gram of dry extract weight. The obtained was 41.4975 ± **0.01 mg of GAE/g dry weight of plant extract.**

5.4 In-Vitro Anti-Inflammatory Assay

5.4.1 Albumin Denaturation Inhibition Assay

The results of this assay were measured by using the percentage inhibition formula. The results showed that with increasing concentrations the percentage inhibition also increases. The value of plant extract was compared with the values of diclofenac sodium which was used as a standard. The results show that the plant extract showed better percentage inhibition as compared to the standard at the following concentrations. The p-value for plant extract was significant (**p value= 0.0021**).

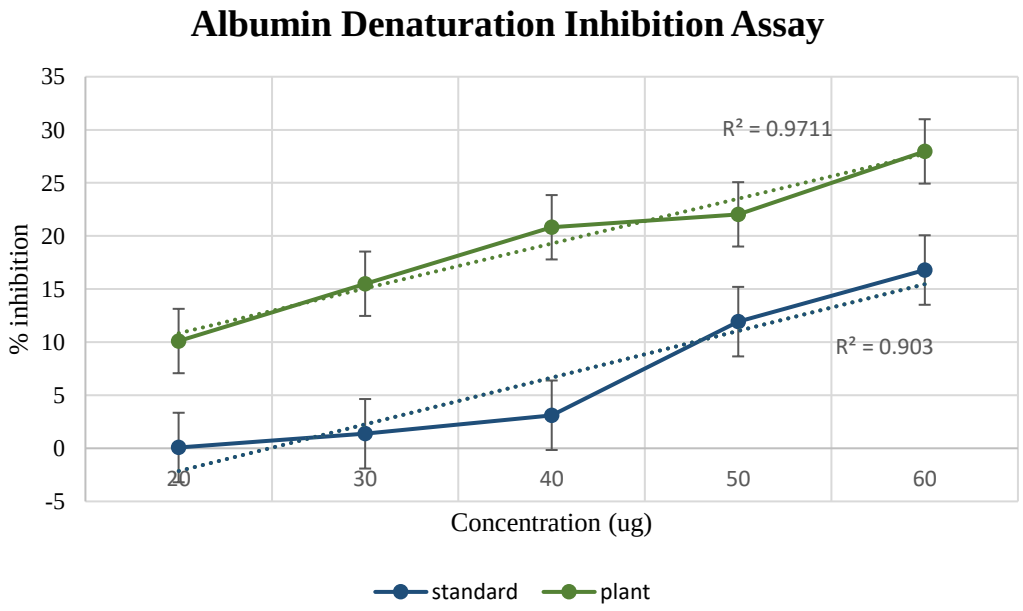

***Figure 22**: Graph for Albumin Denaturation Inhibition Assay*

5.4.2 Membrane Stabilization Assay

The assay was performed to analyze the ability of plant extract to stabilize the lysosomal membranes. The values were calculated by the formula:

$$\text{Membrane Stability} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100.$$

The plant extract showed an increased ability than the standard diclofenac sodium. The values of plant extract were significant with a p value of **0.0101.** The graph shows the ability of plant extract to stabilize membrane increases with an increase in concentration.

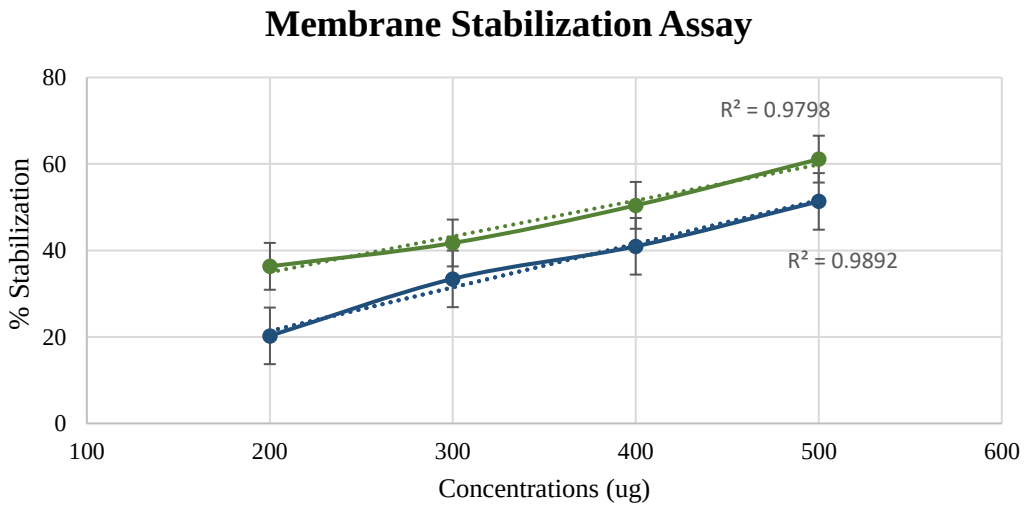

Figure 23: *Graph of Membrane Stabilization Assay*

5.5 Toxicological Assay

5.5.1 In-Vitro Hemolytic Assay

This assay was conducted to assess the toxicological effect of plant extract on red blood cells. Triton-X was used as a positive control as it gives 100% hemolysis. PBS was used as a negative control as it has 0% hemolysis. The values obtained were compared with the positive and negative controls to assess the hemolytic activity of extract. The serial dilution

of extract with concentrations from 0.1ug to 1000ug were taken and percentage hemolysis was calculated using the formula mentioned above. The results showed that the plant extract had no hemolytic activity and was safe to use.

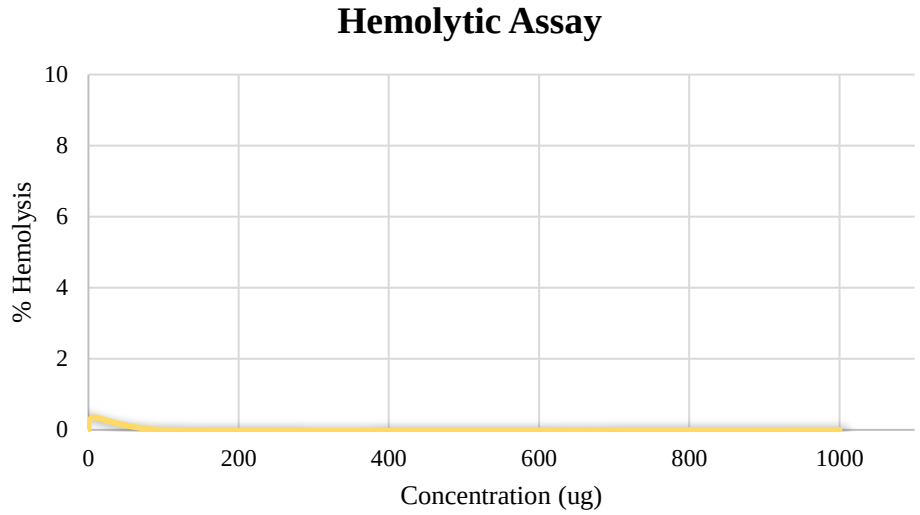

Figure 24: *Hemolytic Activity of Plant Extract*

5.5.2 Acute Toxicity Analysis (LD50)

The lethal dose was not calculated as there was no mortality within the range of 30mg-5000mg/kg in any of the groups. Mice showed no sign of toxicity but other physical changes were observed. The fur of mice became smooth and soft. They were calm throughout the experiment and showed an increase in weight.

PHASE NO.	***DOSE CONCENTRATIONS***	***SIGNS OF TOXICITY***	***MORTALITIES***
Phase 1	30, 300, 500 (mg/kg)	No sign of toxicity	0
Phase 2	1000, 2000, 3000 (mg/kg)	No sign of toxicity	0
Phase 3	5000 (mg/kg)	No sign of toxicity	0

Table 7*: Toxicity Analysis of Thymus serpyllum Extract*

5.6 Microemulsion Formulation

5.6.1 Screening of Components

The oils that were used to check the solubility of plant extract were castor oil and oleic acid. Oleic acid showed better solubility than castor oil hence it was selected as the oil medium. Solubility in other components like Tween 20, and ethanol was also calculated.

Oleic acid is also reported to have anti-inflammatory properties as it plays a role in the activation of immune component cells. Tween 20 is considered as a safe chemical to be used in skincare and is used to emulsify oil and water. Ethanol is used as an anti-infective in topical medications.

5.6.2 Construction of Pseudo-Ternary Phase Diagrams

In order to ascertain the microemulsion regions, pseudo-ternary phase diagrams were constructed using the titration method. The diagrams were constructed between the oil phase (oleic acid), aqueous phase (distilled water) and Smix (Tween 20: Ethanol). Different weight ratios (Km) of Smix were used and the one with the greatest microemulsion area was selected. Km ratios of 1:1, 2:3 and 3:2 (tween 20: ethanol) were used. Oil phase was mixed with Smix in different ratios and water was added slowly. Once the mixture turns turbid, it was marked on the diagram as this was the maximum point for microemulsion formation. The microemulsion regions of different Km ratios are shown in coral color.

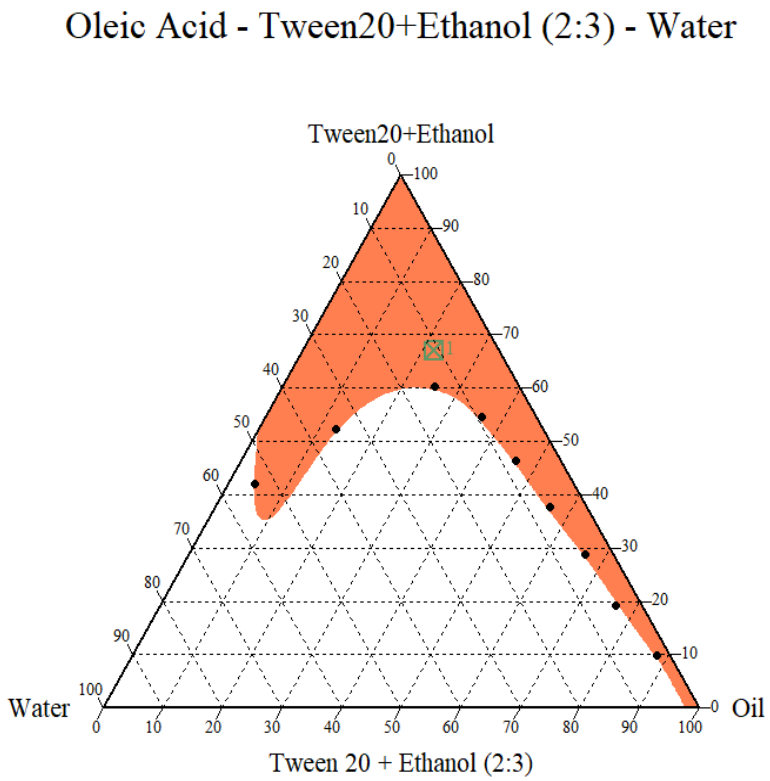

Figure 25: *Pseudo-ternary Phase Diagram of Km ratio (2:3)*

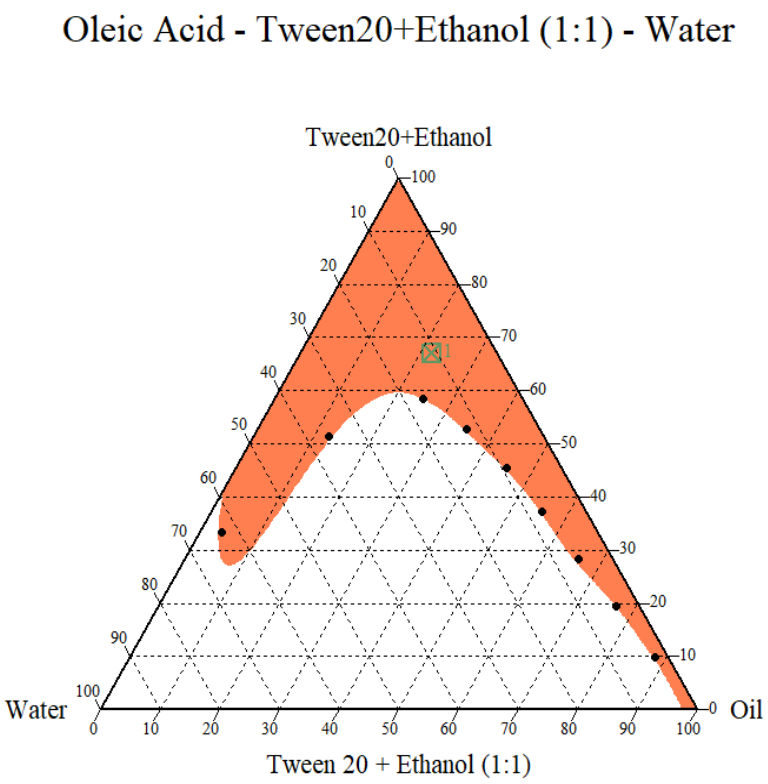

Figure 26*: Pseudo-ternary Phase Diagram of Km ratio (1:1)*

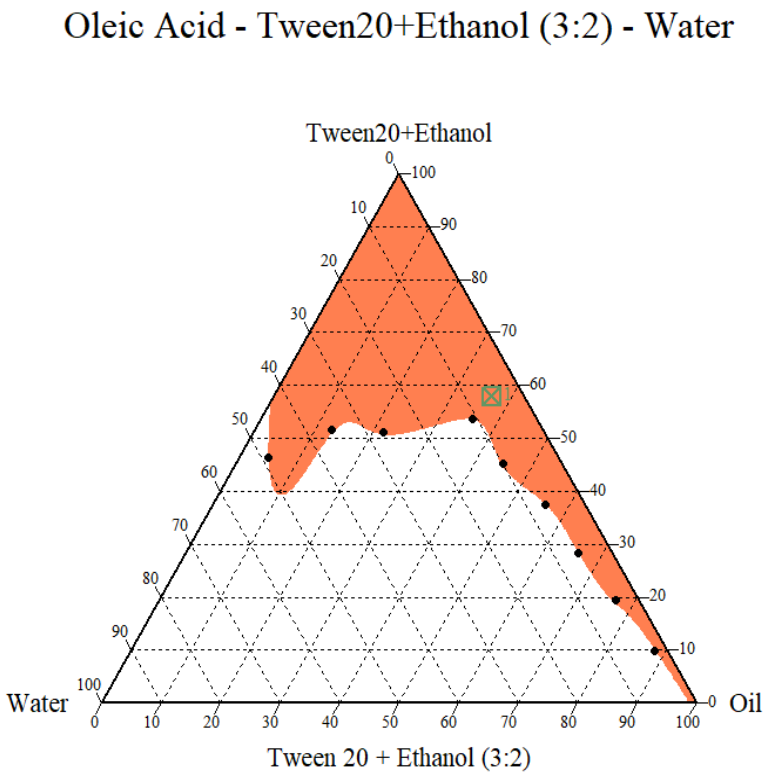

Figure 27: *Pseudo-ternary Phase Diagram of Km ratio (3:2)*

5.6.3 Formulation Development

The pseudo-ternary phase diagrams of Km ratio 1:1 and 3:2 was selected as they showed the most area for the microemulsion region. Different points from the microemulsion regions of the two diagrams were selected and microemulsions were formulated with the given concentrations.

Three points on diagram of km 1:1 was selected and 4 points from diagram km 3:2 were selected for the formulations. Seven microemulsions were formed with different concentrations of oleic acid, tween20, ethanol and water. the formulated microemulsions were than further tested for stability. The selected points are shown with green dots.

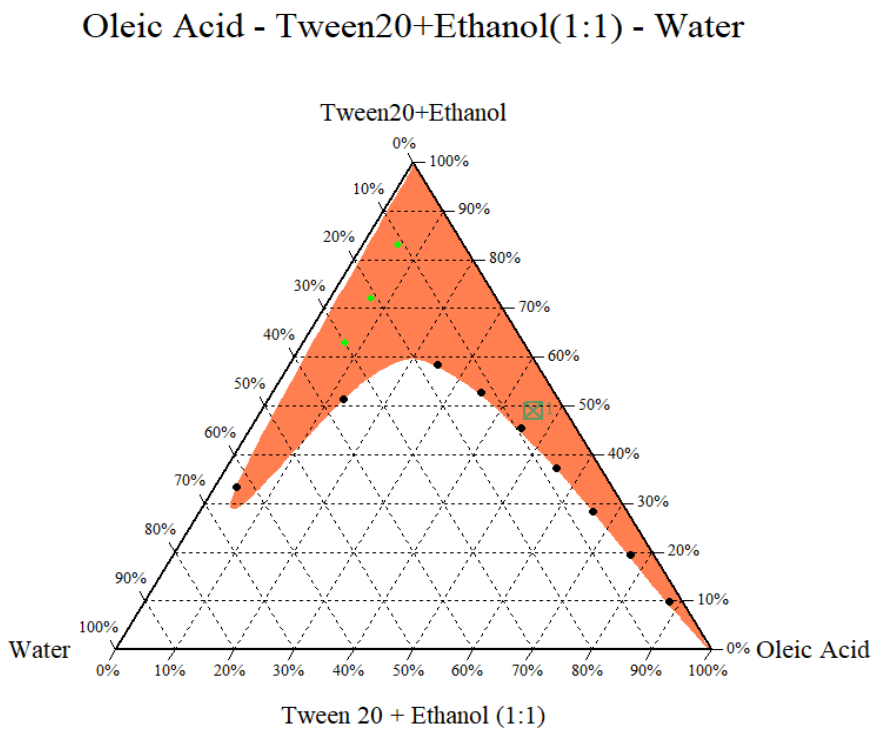

Figure 28: *Selected Points for ME1, ME2 and ME3*

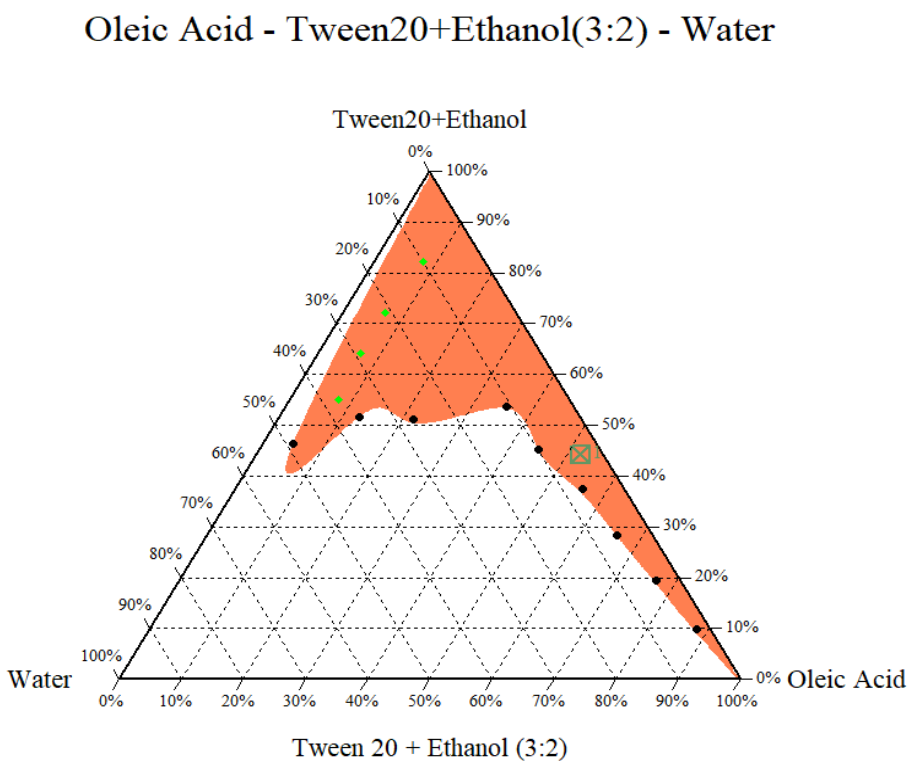

Figure 29: *Selected Points for ME4, ME5, ME6 and ME7*

Microemulsion No.	***Km Ratio***	***%Smix***	***%Oil***	***%Water***
ME1	1:1	83	6	11
ME2	1:1	72	7	21
ME3	1:1	63	7	30
ME4	3:2	82	8	10
ME5	3:2	72	7	21
ME6	3:2	64	7	29
ME7	3:2	55	8	37

Table 8: *Concentrations of Oil, Water and Smix in different formulations of ME*

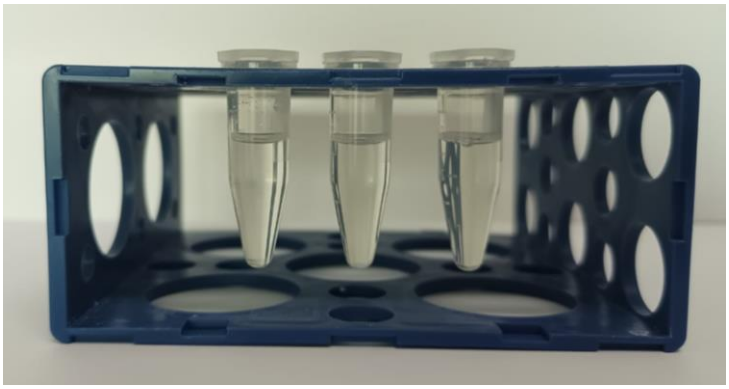

Figure 30: *Formulation ME1, ME2 and ME3 (from left to right)*

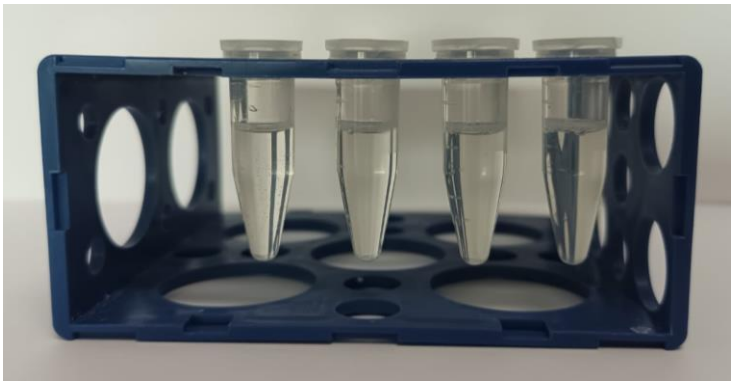

Figure 31: *Formulation ME4, ME5, ME6 and ME7 (from left to right)*

5.6.4 Stability Testing of Formulations

Different Stability tests like Centrifugation, Heating and cooling cycles, dilutions and freeze thaw cycle were conducted to estimate the thermodynamic stability of the seven formulated microemulsions. The microemulsions which showed phase separation were rejected. pH of the formulations was also calculated using pH strips.

	Centrifuge	***Freeze thaw cycle***	***1/10 dilution***	***1/100 dilution***	***Heating cooling cycle***	***pH***
ME1	No phase separation	No phase separation	Clear	Clear	No phase separation	5
ME2	No phase separation	No phase separation	Turbid	Clear	No phase separation	5
ME3	No phase separation	No phase separation	Turbid	Clear	No phase separation	5
ME4	No phase separation	No phase separation	Clear	Clear	No phase separation	5
ME5	No phase separation	No phase separation	Clear	Clear	No phase separation	5
ME6	No phase separation	No phase separation	Clear	Clear	No phase separation	5
ME7	No phase separation	No phase separation	Turbid	Clear	No phase separation	5

Table 9: *Stability Test Results for the Seven Formulated Microemulsions*

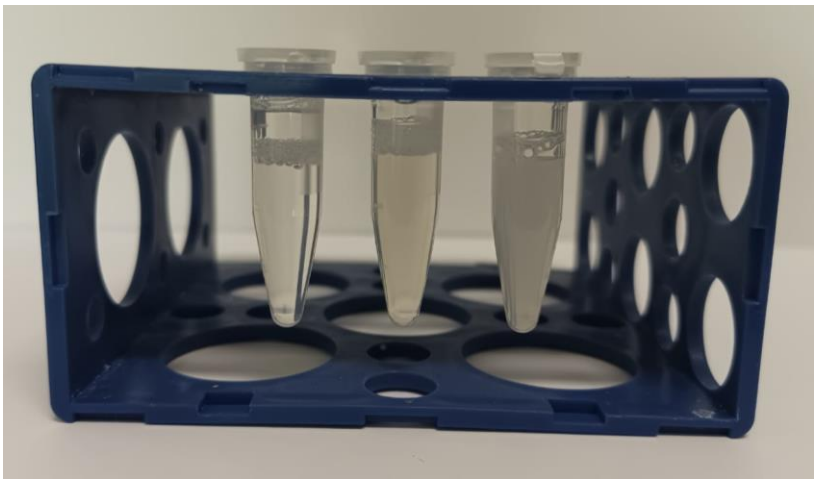

Figure 32: *Dilution test (1/10) for ME1, ME2, ME3*

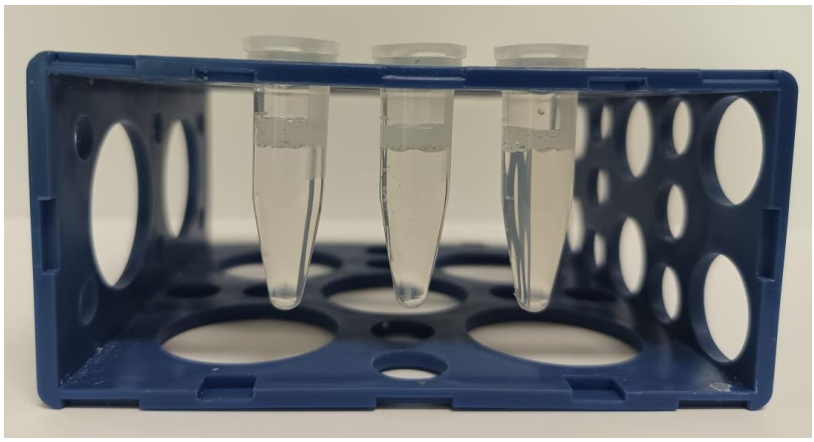

Figure 33: *Dilution test (1/100) for ME1, ME2, ME3*

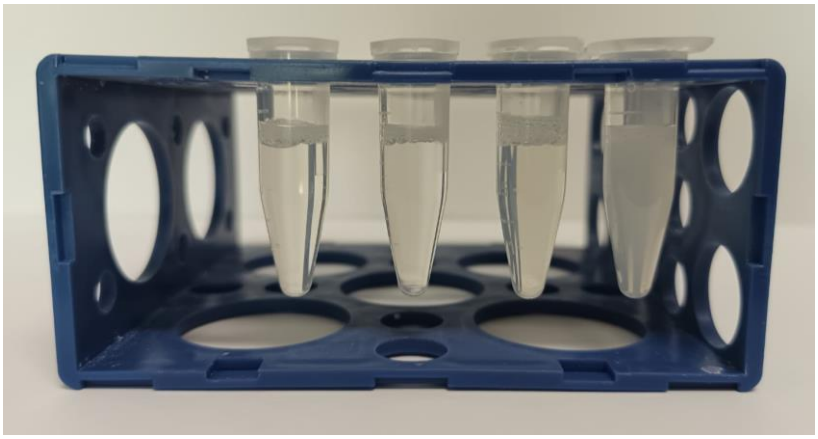

Figure 34:*Dilution test (1/10) for ME4, ME5, ME6, ME7*

Figure 35: *Dilution test (1/100) for ME4, ME5, ME6, ME7*

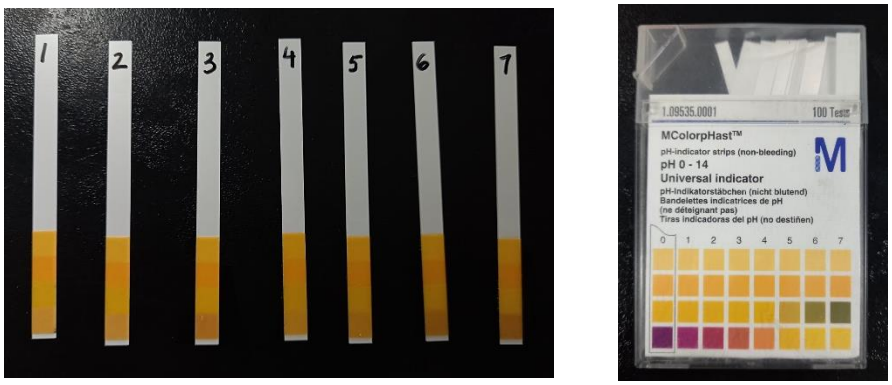

Figure 36*: pH of all seven formulations with key*

5.6.5 Percentage Transmittance of Microemulsion

The percentage transmittance was used to identify the transparency of the formulated microemulsions. The microemulsions which showed percentage transmittance ≥91% were considered clear and transparent as the percentage transmittance value for water was 91.7064% ± 0.6719.

Microemulsion Number	***Percentage Transmittance (%)***
ME1	90.6
ME2	92.2
ME3	91.8
ME4	92.2
ME5	92
ME6	91.3
ME7	65.3

Table 10: *Percentage Transmittance of Microemulsions*

5.6.6 Identification of Type of Microemulsion

5.6.5.1 Dye Solubility

Water soluble dye, Trypan Blue, was used for the identification of type of microemulsion. The dye gave a blue color in the continuous phase indicating the presences of o/w microemulsion.

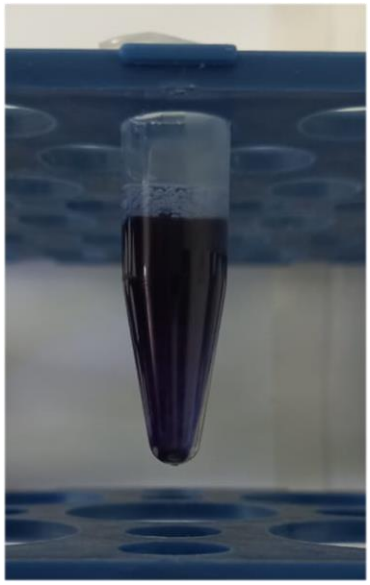

Figure 37: *Dye Solubility Test*

5.6.5.2 Cobalt Chloride Test

The cobalt chloride coated strip turned pink indicating the presence of o/w microemulsion.

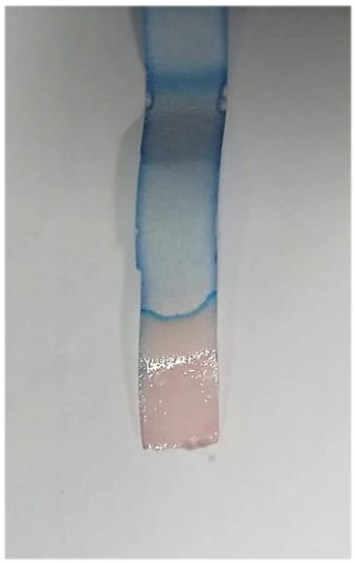

Figure 38: *Cobalt Chloride Strip turned Pink*

5.6.5.3 Creaming Test

After heating the microemulsion upward formation of creaming indicates the presence of o/w microemulsion.

Figure 39: *Upward Creaming of microemulsion*

5.7 Formulation of Microemulsion With Plant Extract

ME1 and ME6 were selected for the preparation of the microemulsion with plant extract. 0.8% of Thymus serpyllum extract was dissolved in both the microemulsions in appropriate amount of oleic acid. Given amount of Smix was added and mixed thoroughly. The mixture was left to equilibrate and then water was added slowly in a drop wise manner.

5.7.1 Characteristics of Plant Embedded Microemulsions

The blank microemulsions (without the plant extract) and plant embedded microemulsion were analyzed for particle size, Zeta potential, PDI, Conductivity and pH.

Microemulsion number 6 was selected based on the obtained results. The microemulsion has a size in the defined range and has a large value of zeta potential which shows better stability. The lower PDI value indicates better uniformity of particles.

	Km	***Plant conc.***	***Size***	***Zeta Potential***	***PDI***	***Conductivity***
ME 1A	1:1		21.09 nm	-11.5 mV	0.422	0.0327 mS/cm
ME 1B	1:1	0.7%	42.675 nm	-8.62 mV	0.333	0.0602 mS/cm
ME 6A	3:2		171.1 nm	-18.1 mV	0.294	0.0336 mS/cm
ME 6B	3:2	0.8%	138 nm	-11.7 mV	0.23	0.224 mS/cm

Table 11: *Characteristics of Empty and Plant Loaded Microemulsions*

5.8 In-Vivo Skin Irritation Studies

The selected ME6 was then used to check for skin irritability by the use of Draize patch test. The microemulsions were given a score of zero as there was not significant change in the skin of mice before and after the application of the formulation. The skin was smooth and the mice showed no signs of irritation.

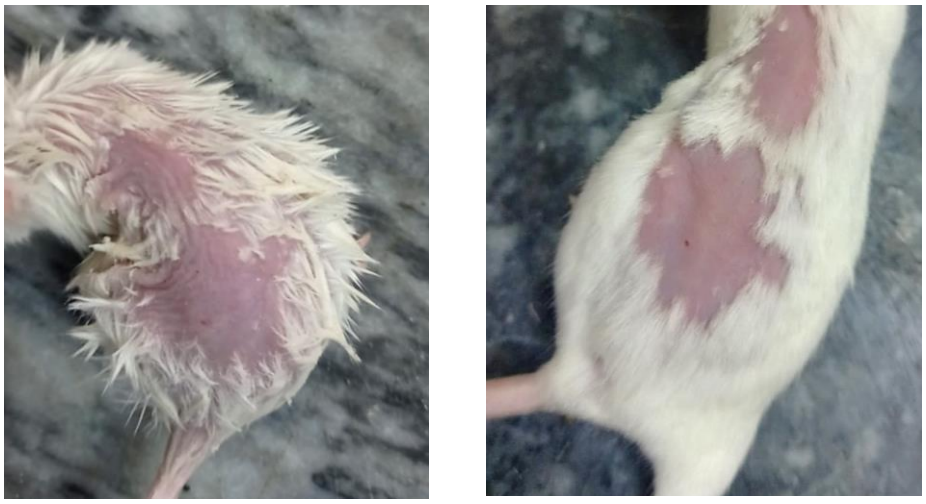

Figure 40: *Skin Irritability Test. The Left Picture Is Before the Application of ME and The Right Picture Is 72 hrs. After the Application Of ME*

5.9 In-vivo Anti-Arthritic Activity of Prepared Microemulsion

5.9.1 Arthritic Induction in Mice

The first step to check the anti-arthritic property of prepared microemulsion was to prepare the arthritic mice models. Collagen Induced Arthritic (CIA) models were prepared. Arthritic induction in the model prepared was validated by the increase in the paw sizes of the mice from day 0 to day 14. Paw measurements were taken in cm^2 and normal and arthritic groups were compared. CIA scoring was also done on the basis of paw swelling and the mice with the CIA scoring of 3 and 4 were taken further for treatment.

There was a significant increase in the paw sizes of arthritic and normal mice with a significance value of $P \leq 0.0001$. The measurements are presented as mean ± standard deviation.

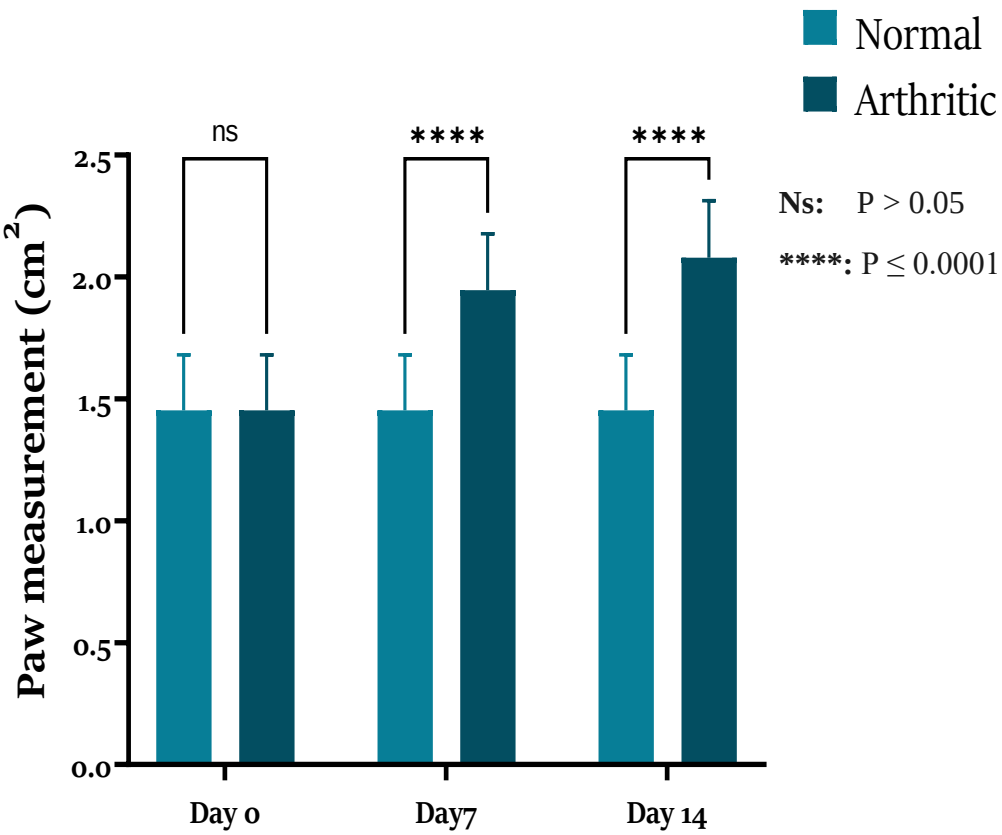

Figure 41: *Graph comparing the paw measurements of normal and arthritic groups*

5.9.2 Anti-arthritic Activity of prepared microemulsion

5.9.2.1 Paw Measurements of Mice Groups During Treatment

To validate the anti-arthritic activity of the prepared microemulsion different parameters were used. Paw measurements were taken before the treatment and after every week at day 0, day 7 and day 14 of the treatment.

Paw measurements (cm^2) of each group were compared at the three days to check the significance of difference between the paw sizes from day 0 to day 14. Negative control and normal group showed no significant difference whereas there was a significant decrease in paw measurements in group 1, group 2 and positive control.

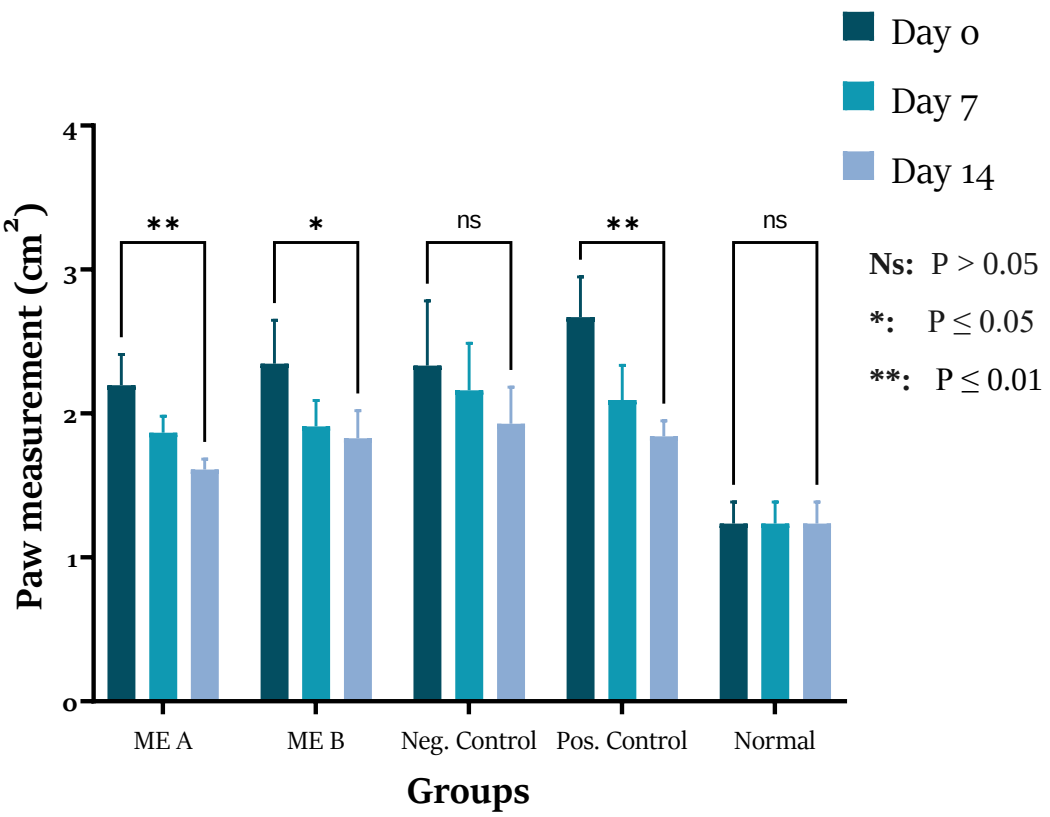

Figure 42: *Comparison between paw measurements of groups before and after treatment*

5.9.2.2 X-rays of Hind Paws

X-rays of left hind paws was conducted to check the joint and soft tissue morphology. The yellow arrows indicate the distance between the joints. And the blue arrows indicate the soft tissue inflammation. There was significant distance between the joints in normal and treated groups, indicating that our treatment helped in limiting the joint destruction. In negative control there were one or two joints fusion which indicates joint and cartilage destruction.

There was no soft tissue inflammation in the normal paws, whereas there was a significant inflammation in both negative and positive controls. The microemulsion groups show less inflammation as compared to the control groups indicating that our microemulsion was effective in reducing inflammation.

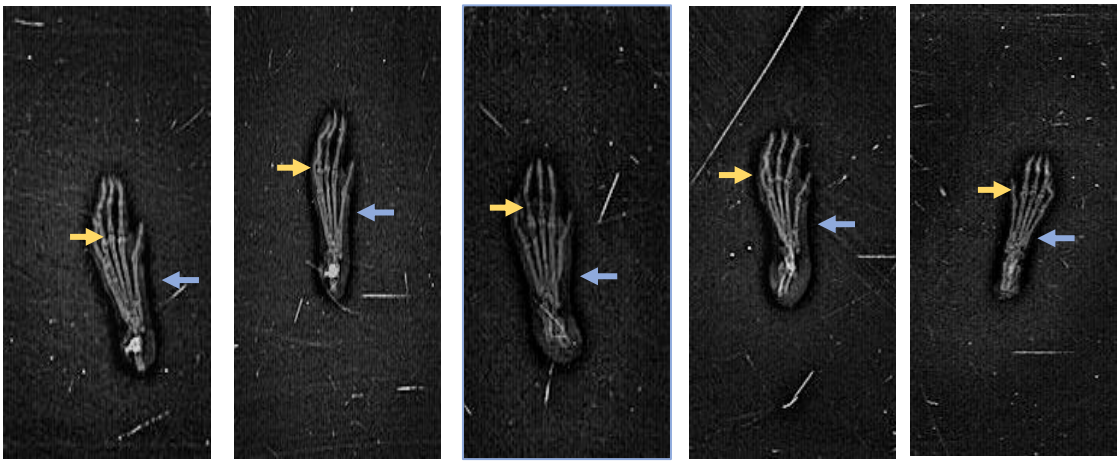

Figure 43: *X-rays of left hind paws. From left to right: Group1, Group2, Group3, Group4 and Group5*

5.9.2.3 Spleen Indices

Spleen indices were calculated as to see the effect of formulated microemulsion on spleen size as during autoimmune diseases the spleen increases in size. There was a significant difference between the values of negative control and normal indicating there was indeed spleen enlargement in arthritic group. The treated groups showed values closer to normal group indicating a protective function.

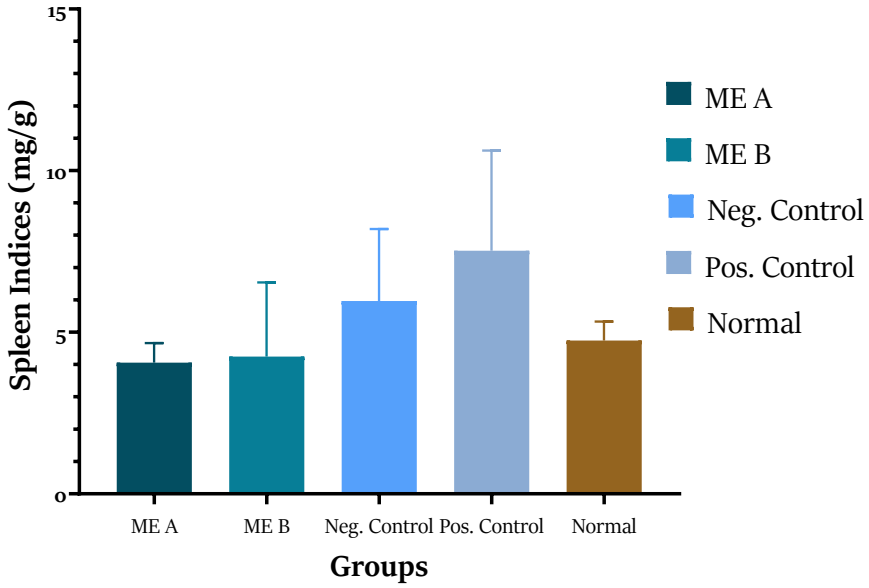

Figure 44: comparison of Spleen indices of different groups

5.9.2.4 Rheumatoid Factor Test

Rheumatoid factor is an autoantibody produced by the B-cells. In RA, the levels of RF increase which trigger the immune system further to cause further damage at the joint site. The normal levels of RF in human are around 0-14 IU/ml serum. Our results showed that there was a significant difference between the serum levels of normal and negative control group. Whereas the levels of group 2, which is plant based microemulsion, are within the normal range. Suggesting that the Thymus serpyllum loaded microemulsion suppressed the release of RF in serum.

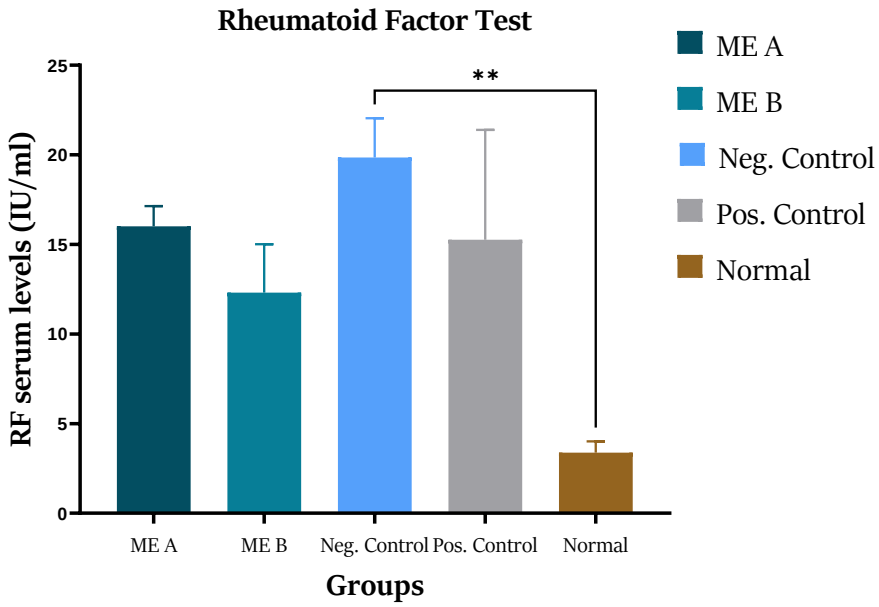

Figure 45 RF serum levels of different groups

CHAPTER 6
DISCUSSION

Rheumatoid arthritis is an autoimmune disease that effects 1% of population worldwide. Several genetic and environmental factors play a role in the onset of the disease. The HLA-DRB1 locus is the most studied genetic factor which produces a 5 amino acids molecule known as the shared epitope which is currently used as a diagnostic marker. In addition to the genetic, the environmental factors like smoke, dust and even the microbiome also play a role in its pathogenesis. There is currently no cure for rheumatoid arthritis but different drugs are used to manage its symptoms and stop its prognosis. These drugs provide therapeutic effect to a limit and their prolong use is associated with numerous side effects like stomach ulcers, kidney failure, heart diseases etc. Due to these limitations of the conventional medications, scientists are looking for alternate compounds present in the medicinal plants to treat Rheumatoid arthritis and other inflammatory diseases. The therapeutic potential of several medicinal plants has been proven through in-vitro and in-vivo experimentation. The results have shown these plants to be safe and non-toxic to be used as medication.

The phytochemicals present in a plant can be classified into three groups, phenolic compounds, terpenoids and alkaloids. These phytochemicals have been investigated to show various biological functions like anti-allergic, anti-inflammatory, anti-bacterial and anti-cancer. Plants have also been an excellent source of many new anti-inflammatory compounds that work by inhibiting the inflammatory pathways of immune system. Inflammation is immune systems response to an injury or an infection. Chronic inflammation is related with the release of inflammatory mediators known as ROS species. It is also involved in the activation of various pro-inflammatory cytokines which are also involved in the pathogenesis of several inflammatory diseases including Rheumatoid arthritis. Phytochemicals have been known to have anti-inflammatory potential which can be used as therapeutic agents for treating inflammatory diseases (Shin SA, 2020).

The objective of the current study was to identify the anti-oxidant and anti-inflammatory potential of *Thymus serpyllum* seed extract to be used as a therapeutic agent for

Rheumatoid arthritis. For this purpose, phytochemical screening of the plant extract was done. The extract was positive for phenols, flavonoids, steroids, anthraquinone, saponins and cardiac glycoside. The phenolic phytochemicals have been researched to show activity against oxidative stress linked chronic diseases (Lin D, 2016). Flavonoids have also been reported to have major anti-inflammatory potential (Mengjie Kong, 2021). Plant based steroids are known to have cardiotonic effect and are also reported to have anti-bacterial and anti-insecticidal properties (Erum Iqbal, 2015).

The quantitative measurements of phenolic and flavonoids was done by calibrating a standard curve. Linear regression was applied and the equation obtained was used to calculate the values. The plant extract showed significant quantity of both phenolic and flavonoid compounds.

To assess the anti-oxidant potential of our plant extract, DPPH assay and FRAP assay were done. The ethanolic extract was efficient in removing the free radicals which can be proved by the results obtained. With an increase in concentration there was an increase in the percentage inhibition of the plant extract activity. The anti-inflammatory action of the plant extract was validated by the use of albumin denaturation inhibition assay and membrane stabilization activity. The plant extract was efficient in inhibiting the heat induced denaturation of albumin and the effect increased with an increase in the concentration. Similarly, the plant showed great membrane stabilization potential which is very important for its role as an anti-inflammatory agent.

The toxicity of plant was assessed to confirm its safety and use as a therapeutic agent. The hemolytic assay results showed the plant extract to have zero percent hemolysis activity at the concentrations of 0.1ug to 1000ug. The in-vivo acute toxicity assay showed no mortality at the highest concentration of 5000mg/kg. Indicating that our plant was safe to use at these concentrations.

Another objective of this study was to make an efficient drug delivery system for rheumatoid arthritis. Topical route was selected over the oral because it provided targeted drug release and less chances of the therapeutic compound to be metabolized by the

stomach enzymes. For this purpose, topical microemulsion embedded with Thymus serpyllum extract was formulated.

The selected components of the microemulsion were oleic acid as the oil phase, ethanol as co-surfactant, tween 20 as surfactant and distilled water for aqueous phase. The microemulsion that had the best characteristics was the ME6 which had 6% oil, 29% water and 64% Smix and was an O/W microemulsion which are best for the topical application. The microemulsion contained 0.8% of dry plant extract of Thymus serpyllum in the oil phase.

Characterization of the formulated microemulsion showed that it has a size of 171 nm when empty and 138nm when loaded with plant extract, which is within the range of microemulsion (10nm – 300nm). The plant based microemulsion had a high negative zeta potential (-11.7mV) which proves that the microemulsion is stable and there are less chances of phase separation over time. It has a less PDI value (0.23) which proves that the particles are uniform in size and has a pH of 4.88 which is ideal for topical administration.

To check the anti-arthritic affect of the prepared microemulsion CIA mouse model where prepared. Arthritis induction is mice were confirmed on the bases of increase in paw measurements throughout the induction period and the mouse were CIA scored on the basis of swelling and inflammation. Mouse were then divided into five groups. Group one was arthritic mice that were given empty microemulsion. Group two was arthritic mice treated with Thymus serpyllum loaded microemulsion. Group three was negative control. Group four was positive control and was treated with leflunomide. Group five was normal healthy mice for comparison.

Our plant based microemulsion showed significant anti-arthritic potential as it showed significant decrease in paw size over the course of treatment. X-rays of hind paws showed that plant microemulsion played a protective role and there was no degradation of joints and cartilage. There was significant decrease in soft tissue inflammation as well. Serum analysis showed that the values of RF serum levels (IU/ml) were within the normal range for group two.

CHAPTER 7
CONCLUSION

The study showed that Thymus serpyllum seed extract have anti-arthritic potential as it possesses anti-inflammatory and anti-oxidant activity which has been confirm by both in-vitro and in-vivo studies. Further studies are also required to validate the efficacy of Thymus serpyllum extract against Rheumatoid arthritis. The microemulsion created proved to be an effective drug delivery system for topical application. This research would help in the advancement of therapeutic development for Rheumatoid arthritis.

CHAPTER 8
FUTURE PROSPECTS

The Thymus serpyllum extract showed great anti-oxidant and anti-inflammatory activity. Hence its potential as a therapeutic agent in other inflammatory diseases can also be identified. The prepared microemulsion can be further tested on larger scale to validate its potential as an effective drug delivery system.

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