Investigation of Therapeutic Potential of *Thymus serpyllum* on Inflammation and Autophagy in Collagen Induced Arthritic Mice

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Copyright Statement

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Dedicated to those who taught me

Abstract

Rheumatoid Arthritis (RA) is a systemic autoimmune disorder that affects approximately 1% of the world population and characterized by bone erosion, cartilage degradation, synovial hyperplasia, inflammatory and fibroblast like synoviocytes (FLS) infiltration. In rheumatoid arthritis, macroautophagy has been observed to be elevated which complicates its pathogenesis and treatment. As resistance to current treatment options is a major concern with disruption of autophagy being as one of the primary reasons to the resistance, to shed light on this phenomenon, current study has been carried out. Tomorou, *Thymus serpyllum*, is an indigenous ethnobotanical medicinal plant from HunzaNagar Valley and used against inflammatory problems. In the given study, antiinflammatory and anti-rheumatic effect of 'tomoru' to unravel the underlying mechanism of RA has been evaluated. The results demonstrate that the aqueous and ethyl acetate extracts of tomoru had anti-inflammatory effect as IL6 levels were lowered after their administration to the Collagen Induced Arthritic (CIA) Mice Model. A distinctive pattern in the expression of autophagy and apoptotic markers was observed that indicated that autophagy in RA is via ULK1 independent non-canonical pathway. The extract administration alleviated the symptoms of RA either through induction of ULK-1 dependent pathway or apoptosis induction. Results of immunohistochemistry and scanning electron microscopy of muscle has showed activity tomoru against autophagy while histopathology of ankle and tarsal joints has depicted retention of joint architecture in CIA mice model. This understanding in autophagy in RA has contributed to better disease control and management.

Keywords: Rheumatoid Arthritis, *Thymus serpyllum*, Autophagy, Apoptosis, ULK-1

Abbreviations

%	Percent
°C	Degree Celsius
ACPA	Anti-Citrullinated Protein Antibodies
APRIL	Proliferation Inducing Ligand
axSpA	ankylosing spondylitis
BLyS	B-Lymphocyte Stimulator
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CCL21	Chemokine C-C motif ligand 21
CCP	Cyclic Citrullinated Peptide
CMV	Cytomegalovirus
CNS	Central Nervous System
COX	*cyclooxygenase*
CRP	C-reactive protein
CTAB	Cetyltrimethylammonium bromide
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DAS28	Disease activity scoring system
DISC	Death-Inducing Signaling Complex
DMARDs	Disease Modifying Anti-rheumatic Drugs
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphate or deoxynucleotides
DPPH	2, 2,-Di-Phenyl-2-Picryl Hydrazyl Hydrate
DRAM	Damage-Regulated Autophagy Modulator
E.coli	*Escherichia coli*
EBV	Epstein Bar Virus (EBV)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	et alia
FcgR	Fc receptor for IgG
FCGR2A	low affinity immunoglobulin gamma Fc region receptor IIa
FLS	Fibroblast-Like Synoviocytes
FLS	Fibroblast Like Synoviocyte
Foxp3	Forkhead box P3
g	g
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
H_2O	Water
HIF	Hypoxia Inducible Factor

HLA	Human Leucocyte Antigen
HPA	Hypothalamic-Pituitary-Adrenal
IAPs	Inhibitors of Apoptosis Proteins
Ig	Immunoglobulin
IL	Interleukin
kb	Kilo base
KV	Kilovolt
LDL	Low Density Lipoprotein
M	Molar
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
min	Minute
ml	milliliter
ML	Maximum Likelihood
mM	millimolar
MMP	Matrix Metalloproteins
MPO	Myeloperoxidase
MRI	Magnetic Resonance Imaging
NaCl	Sodium Chloride
NET	Neutrophil Extracellular Trap
NF H_2O	Nuclease free water
NFATc1	Nuclear Factor of Activated T cell cytoplasmic 1
NFκB	Nuclear Factor κ B
ng	nanogram
NK cells	Natural Killer cells
nm	nanometer
NOD	Nucleotide-binding Oligomeriation Domain
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OA	Osteoarthritis
OD	Optical Density
OPG	Osteoprotegerin
PAD	*Peptidylarginine deiminase*
PCR	Polymerase Chain Reaction
PRDM1	Positive Regulatory Domain I element of β-INF gene promoter
PRKCQ	Protein Kinase C theta
PsA	Psoriatic Arthritis
PTPN22	Protein Tyrosine Phosphatase Non-receptor Type 22
PUMA	p53 Upregulated Modulator of Apoptosis
RA	Rheumatoid arthritis
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor Activator of Nuclear Factor κ B Ligand
rbcL	Ribulose bisphospahte carboxylase Large region

RF	Rheumatoid Factor
ROS	Reactive Oxidative Specie
rpm	Round per minute
RT	Room Temperature
SDF-1	Stromal Cell-Derived Factor 1
SDS	Sodiumdodecylsulfate
SNP	Single Nucleotide Polymorphism
ssDNA	single stranded DNA
STAT	Signal Transducer and Activator of Transcription
TAE Buffer	Tris-Acetate-EDTA Buffer
TAGAP	T-cell activation RhoGTAPase Activating Protein
TCR	T Cell Receptor
TE Buffer	Tris-EDTA Buffer
TGF	Tumor Growth Factor
Th	T-helpler
TLR	Toll-Like Receptors
TNF	Tumor Necrosis Factor
TNFAIP3	TNF-α Induced Protein3
TNFRSF14	TNFR super family member 14
TNFSF13	TNF super family member 13
TRAF1	TNF Receptor Associated Factor 1
TRAIL	TNF-related apoptosis-inducing ligand
TRAP	Tartrate-Resistant Acid Phosphatase
TrAP	Transcriptional activator protein
Treg	Regulatory T cells
Tris	Trisma
Tris-HCl	(hydroxymethyl)aminomethane- Hydrochloride
TSP-1	Thrombospondin 1
UV	Ultra-violet
V	volt
VCAM-1	Vascular Cell Adhesion Molecule 1
Wnt	Canonical Wingless
α	alpha
β	beta
µg	micro gram
µl	microliter

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CHAPTER 1 : INTRODUCTION

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA), a multifactorial autoimmune disorder, is characterized by progressive bone erosion and deformation. The root cause of this multifactorial disorder is yet to be explored as both genetic and environmental factors both contribute to the initiation and progression of the disorder. Bone erosion is one of the typical features of RA that begins in the early stages of the disorder and prevails with the progression. Systemic and articular bone loss and thinning is associated with functional disability and risk of fractures (Gough *et al.*, 1994; Schett & Gravallese, 2012; Scott *et al.*, 2000). There is a balanced maintained between bone resorption and formation, which in particular is altered in RA due to the action of pro-inflammatory cytokines and chemokines that hinder in the functioning of osteoblasts and promote osteoclast functioning (Gravallese *et al.*, 2015; McInnes & Schett, 2011).

During RA the immune cells lose self-tolerance and start attacking body's own tissues especially joints. In the course of tolerance breakage auto-antibodies particular to RA prognosis such as anti-citrullinated protein antibodies (ACPAs) and Rheumatoid Factor (RF) are produced. The presence of these auto-antibodies is a preclinical feature of RA. Citrullination, is conversion of arginine to citrulline in peptidylarginine deiminase (PAD) enzyme (Vossenaar *et al.*, 2003). This post-translational modification alters the charge of protein thereby developing neo-antigens that instigate the subsequent auto-antibody production. Presence of neo-antigens and citrullinated proteins evoke an immune reaction against the normal cells of body and produce the disease specific signs and symptoms.

The dysregulation of immune system leads to the infiltration of lymphocytes along with pro-inflammatory cytokines into the synovial tissues leads to progressive deformation and destruction and other arthritic symptoms. Infiltration of lymphocytes, especially phagocytes and dendritic cells, is essential for instigation and pathogenesis

of RA (Lundy *et al.*, 2007). These macrophages and dendritic cells are involved in antigen presentation, maintenance of immunity, T-cell homeostasis and synovial inflammation (Farache *et al.*, 2013; Galli *et al.*, 2011; Geissmann *et al.*, 2010) as circulating activated monocytes, expressing Toll-Like Receptors (TLRs), move into the synovial cavity where the differentiate into macrophages which produce enzymes, cytokines and chemokines that contribute to the destruction of cartilage, bone and synovial inflammation (Choy & Panayi, 2001; Q. Huang *et al.*, 2007; Q. Huang & Pope, 2010; van den Berg & van Lent, 1996).

1.2 Prevalence of Rheumatoid Arthritis

RA, being an autoimmune disorder with poor disease management has become a substantial burden for society and individual (Cross *et al.*, 2014) as there are musculoskeletal disability, declined physical function, medical cost, impaired life quality and decreased societal participation (Kitas & Gabriel, 2011; Sokka *et al.*, 2010). 0.5-1% of world population is affected by the disorder (Lawrence *et al.*, 1998). In United States around 15% of population is affected by RA and studies suggest that native Americans are prone to disorder (Studenic *et al.*, 2012). In Pakistan it is estimated that around 0.55% of population is affected by RA (Akhter *et al.*, 2011; Alam *et al.*, 2011; Hameed *et al.*, 1997).

1.3 Pathophysiology of Rheumatoid Arthritis

1.3.1 Autoimmune Response

RA is a pathologically diverse disorder with the presence of auto-antibodies that stimulate joint swelling and damage (Aletaha *et al.*, 2015; Gonzalez *et al.*, 2008; Honda & Littman, 2012; Scher *et al.*, 2015; Van Gaalen *et al.*, 2004). ACPAs form complex with citrulline containing neo-antigens and self-proteins such as Type II collagen, Vimentin, fibronectin, histone, fibrinogen and α-enolase with RF binding that results in complement activation (Anquetil *et al.*, 2015; Sabharwal *et al.*, 1982; Zhao *et al.*, 2008). Epitope diversity and concentration of ACPAs increase along with inflammatory cytokines, thereby increasing the complications although the origin of these responses is not known. B-cells that produce ACPA are present in the synovium

and these circulating ACPA can be IgM, IgA or IgG isotype with altered glycosylation which has enhanced Fc-receptor and neo-antigen binding (Kerkman *et al.*, 2015; Rombouts *et al.*, 2013; Rombouts *et al.*, 2015). Bone loss can be as a result of ACPA that either activate macrophages through Fc engagement and Toll-like receptor (TLR) ligation via antigen binding or activate osteoclast via binding of cirtullinated vimentin, Fc-receptor engagement and formation of immune complexes (Harre *et al.*, 2012). RF, on the other hand, has direct involvement in macrophage activation and cytokine production (Anquetil *et al.*, 2015; Sokolove *et al.*, 2014). T-cell engagement in autoimmune responses is still to be explored, however, T-helpler 1 (Th) cells have been detected in patients with RA (James *et al.*, 2014).

1.3.2 Inflammation

Synovitis, also known as joint swelling, indicates the inflammation in synovial membrane due to leucocyte infiltration including innate and adaptive immune cells like dendritic cells, monocytes and mast cells, Th-1 cells, Th-17 cells B-cells, plasma cells and plasmablasts. Complex network of cytokines and chemokines develop an inflammatory milieu. Cytokines and chemokines like granulocyte-monocyte colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), Interleukin-1 (IL), IL-6 and other lymphokines induce and aggravate inflammatory responses that attract other immune cells that accumulate in the synovial cavity that leads to bone erosion and synovitis (Feldmann & Maini, 2008). Expression of receptor activator of nuclear factor κ B ligand (RANKL) on the surface of fibroblast-like synoviocytes (FLS), T-cells and B-cells attracts macrophages and dendritic cells with RANK receptor trigger osteoclasts that in turn lead to bone erosion (Pettit *et al.*, 2001; Redlich *et al.*, 2002). Inflammatory cytokines initiate cartilage damage and degradation via matrix metalloproteins (MMPs), binding of cytokines lead to the downstream signaling of inflammatory events in the synovial cavity (Martel-Pelletier *et al.*, 2001).

1.3.3 Bone Erosion

Bone erosion can be detected through radiographic imaging as there is loss of cortical and trabecular bone (Sharp *et al.*, 1971). Although bone erosion is found in many of the bone related disorders such as gout, psoriatic arthritis (PsA),

osteoarthritis (OA) and ankylosing spondylitis (axSpA) but evaluation of bone erosion is distinct for each case as in there are over-hanging edges of bones in gout, the radiographs explicitly demonstrate the pattern of bone erosion thus making classification easier as explained in Table 1.1 (Aletaha *et al.*, 2010; Gladman, 2006; Kay & Gravallese, 2013). Pattern of erosion can be a prognostic feature as the erosive changes lead to cases of disability and mortality (Scott *et al.*, 2000).

1.3.4 Bone Homeostasis

There is a balance between osteoclast and osteoblast activity under normal conditions that maintains bone homeostasis. In RA, bone loss and erosion occurs due to imbalance between the activity, differentiation and impaired function of osteoblasts and osteoclasts as a result of persisting joint inflammation (Baum & Gravallese, 2014). Osteoclasts express calcitonin, tartrate-resistant acid phosphatase (TRAP) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1) through which osteoclasts attach to bone surface and via the action of proton pumps osteoclasts generate acidic environment that lead to bone demineralization. Osteoclasts starts degradation of organic bone matrix leading to bone resorption through the action of MMPs and cathepsin K (Teitelbaum & Ross, 2003). Osteoblasts express transcription factors as that of Runx and osterix that differentiate either through bone morphogenetic protein (BMP) or canonical wingless (Wnt) signaling cascade (Monroe *et al.*, 2012; Nakashima *et al.*, 2002; Sánchez-Duffhues *et al.*, 2015; Schroeder *et al.*, 2005). During maturation phase, osteoblasts become proficient in bone matrix mineralization and formation. These osteoblast differentiate into osteocytes that detect mechanical stress and initiate a feedback loop for bone homeostasis and there is a self-regulatory mechanism between osteoblast and osteoclast (Robling *et al.*, 2008).

RANKL is expressed on osteoblast surface which binds to RANK on osteoclast cells that induce differentiation and function (Lacey *et al.*, 1998). Bone resorption and osteoclastogenesis are halted as osteoprotegerin (OPG) binds to RANKL instead of RANK, thereby maintaining homeostasis (Simonet *et al.*, 1997). Pro-inflammatory cytokines like Tumor necrosis factor (TNF), IL-6, IL-1 and IL-17

induce RANKL that induce osteoclast differentiation that lead to bone erosion (Brennan & McInnes, 2008).

1.3.5 Extra-articular Manifestations

Extra-articular manifestations such as interstitial lung disease and vasculitis comes as a result of disease prognosis (Hurd, 1979). Chronic RA is associated with lymphoma, cardiovascular disorders, secondary amyloidosis and mortality which are reduced with treatment, however, methotrexate and TNF inhibitors induces nodulosis that is not different from rheumatic nodules and psoriasis-like lesions respectively (Choi *et al.*, 2002; Jacobsson *et al.*, 2007; H. H. Lee *et al.*, 2007; Listing *et al.*, 2013; Patatanian & Thompson, 2002).

1.3.6 Autophagy and Apoptosis

Rheumatoid Arthritis (RA) a systemic autoimmune disorder characterized by joint inflammation and bone degeneration along with infiltration of fibroblast-like synoviocytes (FLS) that are resistant to apoptosis. Resistance to apoptosis is achieved through various mechanisms, of which increased autophagy holds prime importance as studies have revealed that rate of apoptosis is in inverse relation with LC3 and Beclin-1 (Cuda *et al.*, 2016; Gordy *et al.*, 2011). Autophagy and apoptosis are two interlinked and highly regulated processes that determine the fate of a cell. Apoptosis, the programmed cell death, acts either through extrinsic or intrinsic pathway involving the interplay of caspases that are determinant factors for the execution of pathway. Binding of ligands to death receptor leads to death-inducing signaling complex (DISC) formation and recruitment that initiate caspase 8 that activates further signal transduction (Thome & Tschopp, 2001). Whereas autophagy, the self-degradative mechanism involves autophagosome formation in which a damaged organelle is trapped and lead to degradation. ATG genes and Beclin-1 are involved in promoting autophagy after a stimulus which can be a citrulinated protein as in case of RA (Dai & Hu, 2015).

In case of RA autophagy induction is mostly due to ER stress and proteasome inhibition (Kato *et al.*, 2014). Autophagy acts as an adaptor between cell death and

survival thus is a cytoprotective and cytodestructive process in RA synovial fibroblasts (Allan & Clarke, 2009; Buckland, 2013; Kato *et al.*, 2014). Resistance to methotrexate treatment is due to increased autophagy in the cells thus making it a huge concern (Xu *et al.*, 2015). Citrullination and antigen presentation that lead to T-cell activation are regulated by autophagy (Ireland & Unanue, 2011; Scally *et al.*, 2013). Moreover studies suggest that citrullinated protein production is due to increased autophagy, thus implying that autophagy plays vital role in RA prognosis (Sorice *et al.*, 2016).

1.4 Causes of Rheumatoid Arthritis

1.4.1 Genetic Causes

Disease severity, prognosis and treatment success are attributed to the genetic predisposition due to single nucleotide polymorphism (SNP) in certain genes. Of these human leucocyte antigen (HLA) also known as major histocompatibility complex (MHC), present on chromosome 6 region 6p21.31, are of prime importance as it has been observed that RF or ACPA positive patients possess HLA-DRB1 locus. HLA-DR forms complex with T cell receptor (TCR) for recognition and antigen presentation. For the proper functioning of immune system a regulated expression of HLA genes is required. All the three classes of HLA are present on chromosome 6 and have different roles. One of the gene is inherited paternally whereas the other maternally.

Studies have shown association of protein tyrosine phosphatase non-receptor type 22 (PTPN22), cytotoxic T-lymphocyte antigen 4 (CTLA4), protein kinase C theta (PRKCQ), AFF3, IL-2RA, IL-2, IL-21, CD40, CD28, Fc receptor for IgG (FcgR), PAD4, signal transducer and activator of transcription (STAT) 4, TNF-α induced protein3 (TNFAIP3), T-cell activation RhoGTAPase activating protein (TAGAP), REL, Chemokine C-C motif ligand 21 (CCL21), positive regulatory domain I element of β-INF gene promoter (PRDM1), TNF receptor associated factor 1 (TRAF1) BLK, low affinity immunoglobulin gamma Fc region receptor IIa (FCGR2A), TNFR super family member 14 (TNFRSF14) and others in RA (van der Helm *et al.*, 2005).

1.4.2 Environmental and Epigenetic Factors

Epigenetic modifications, environmental factors along with personal habits may lead to initiation of autoimmunity and RA.

1.4.3 Smoking

RA has a complex chemistry with environment and genes thus bronchial stress caused by smoking and other reasons make a person prone to the disease. HLA-DR4 acts synergistically with smoking and increase the risk for ACPA possession (Klareskog *et al.*, 2006). Environmental stressors provoke PAD4 that citrullinate mucosal proteins and produce neo-epitopes that lead to loss of tolerance and ACPA autoantibody production that are detected by anti-cyclic citrullinated peptide (CCP) assay (De Rycke *et al.*, 2004; Vincent *et al.*, 1999). Around 44-64% of RA patients with citrullinated proteins and ACPA have association with smoking PTPN22 and HLA-DRB1*04 (Mahdi *et al.*, 2009).

1.4.4 Infectious Agents

Many infectious agents such as *Epstein Bar Virus* (EBV), *cytomegalovirus* (CMV), *E.coli* and heat shock proteins are linked with RA but their exact mode of action remains a mystery to be resolved. There is a possibility that either they mimic or resemble self-peptides and lead to production of auto-reactive RF, ACPA, T and B cells. Certain bacterial species are known to cause septic arthritis. Amongst bacterial species, *Staphylococcus aureus* is most common cause for septic arthritis, cases have been reported from Europeand United States of America (Belkhir *et al.*, 2014; Mathew & Ravindran, 2014). *Haemophilus influenzae, S. aureus, Kingella kingae, Shigella, Campylobacter, Yersinia* spp. and *Salmonella* have also been reported to be associated with bacterial arthritis in children, younger and older patients (Gupta *et al.*, 2003; Wang *et al.*, 2003).

1.4.5 Hypothalamic-Pituitary-Adrenal (HPA) Axis

The HPA-axis activates increased glucocorticoid production during inflammation under the influence of IL-6, IL-1β and TNF-α. The increased glucocorticoids convert acute arthritis to chronic phase and this phenomenon is under

aggressive investigation (Alten & Wiebe, 2015). Central Nervous System (CNS) is involved in immune regulation through the production of neurotransmitters. HPA-axis regulate inflammation through cytokine profile and the molecular pathways involved specifically to RA is under study.

1.5 Diagnosis of RA

1.5.1 Blood Test

Clinical and pre-clinical diagnosis of RA includes blood tests. The auto-antibodies formed against neo-antigens can be detected in the serum of patients even prior to the onset of the disorder (Sokolove *et al.*, 2012). ACPAs and RF produced are responsible for the establishment of chronic disorder. C-reactive protein (CRP) is also a marker for RA pre-clinical diagnosis.

1.5.2 Imaging Scans

Imaging techniques like magnetic resonance imaging (MRI), X-rays and ultrasound are used to visualize bone damage and erosion. Bone damage might result in narrowing of the joint spaces which can be detected via imaging techniques.

1.5.3 DAS28 Scoring System

Disease activity scoring system (DAS28), is used to evaluate the disease upon counting of 28 joints that comes under the umbrella of RA. Physicians evaluate the inflammation and degree of inflammation in joints out of 28 along with blood profile against CRP and ACPAs Figure 1.1. Criteria for RA classification is summarized in Table 1.1.

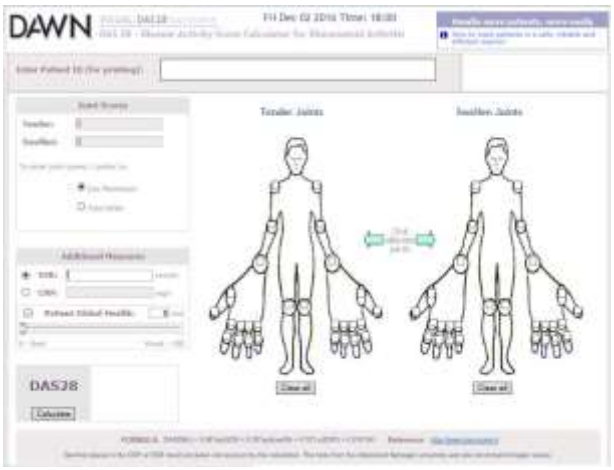


Figure 1.1:DAS28 Scoring System calculator to determine RA.

Table 1.1: Classification criteria for RA from A-D. (Aletaha *et al.*, 2015)

Criteria	**Score**
A. Joint Inflammation	
1 Large Joint	0
2-10 Large Joints	1
1-3 Small Joints	2
4-10 Small Joints	3
>10 Joints	5
B. Serology (at least one should be positive)	
Negative for RF and ACPA	0
Low Positive for RF and ACPA	2
High Positive for RF and ACPA	3
C. Acute Phase Reactants (at least one should be positive)	

Normal CRP and ESR	0
Abnormal CRP and ESR	1
D. Duration of Symptoms	
<6 Week	0
>6 Week	1

1.6 Treatment of RA

Recently, many molecules have been opted as therapeutic targets for RA. There is no cure for RA but options for better disease management. Some treatments are meant to alleviate the arthritic symptoms whereas others just slow the disease progression.

1.6.1 Non-steroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs are meant to relieve pain only and are also known as analgesics. NSAIDs target and block *cyclooxygenase* enzymes, COX-1 and COX-2 that are responsible for pain and fever. Usually NSAIDs are used to treat acute inflammation as in case of certain allergies, fever and moderate pain.

1.6.2 Steroids

Steroids are generally referred as corticosteroids that are prescribed in initial stages of autoimmune disorder. Corticosteroids mimic the mechanism of cortisol hormones produced by adrenal glands. These slow down the prognosis of the disorder by reducing inflammation, pain and joint distortion. Dexamethasone and prednisone are commonly prescribed immune-suppressant steroids.

1.6.3 Disease Modifying Anti-rheumatic Drugs (DMARDs)

DMARDs usually slow down disease progression and protect against permanent damage. Inflammation and further damage is decelerated or halted as DMARDs act on and modify autoimmune cells that are responsible for disease complications. Commonly used DMARDs include hydroxychloroquine, leflunaminde and methotrexate.

1.6.4 Biologics

Biologics are a new class of drugs that are being used to cure RA. These are meant to halt the action of certain factors such as inflammatory cytokines that are actively involved in disease progression and associated complications. IL-1, TNF, T-cell and B-cell blockers have been extensively studied and some of them are in market as a treatment option.

1.7 Ethno-botanical Medicine in RA

Plants contain phytochemical that play a crucial role as being immunoboosters and immunomodulators. These active compounds have medicinal value and are being used to cure diseases for centuries, like roots and leaves of *Capparis spinosa L* of family Caparaceae have medicinal value but only leaves have anti-rheumatic effect (Stickel *et al.*, 2000). Menthol, the essential oil of spearmint of family Lamiaceae has local anti-rheumatic effect (Benoit *et al.*, 1976). The latex produced by plants of family Euphorbiaceae produces efficient anti-rheumatic effect than other parts. Flavonoids from genus Erucaria of family Brassicaceae and terpenoids from Lamiaceae have shown anti-rheumatic activity (Marzouk, 2016). Betula pendula leaves have more terpenoid and flavonoid content than other parts and elicit effective and efficient anti-rheumatic effect (Fischer† r & Seiler, 1961). Ethnobotanically rheumatism can be cured using plants from Brassicaceae (Kirtikar & Basu, 1975).

1.8 *Thymus serpyllum*

Thymus serpyllum is locally known as ‘tomoru’ or wild thyme and is indigenous plant of Himalayan range. It belongs to family Lamiaceae and genus

Thymus that have significant ethnobotanical importance. It is a plant that grows on high altitude, stony ground and is 4 to 7cm in height with flowering season of May-September. Wild thyme constitute of essential oils that possess healing properties against various diseases. Traditionally dried leaves and flowers of the herb are used to prepare tea and infusions against cold, bronchitis, fever and cough (Jabeen *et al.*, 2015). The plant has been explored for its anti-tumor, anti-oxidant, anti-microbial and cytotoxic properties against multi-drug resistance, cancer and other disorders (Jarić *et al.*, 2015).

1.9 Scope of Study

The study aims to assess the anti-rheumatic and anti-autophagic potential of *Thymus serpyllum* in collagen induced arthritic mice models.

CHAPTER 2 : LITERATURE REVIEW

2.1 Rheumatoid Arthritis

RA is a systemic autoimmune disorder that affects joints and has undetermined ethology and causes that complicate the diagnosis and treatment. Systemic complications, progressive disability and early death are the consequence of the disorder, however, with the advancement new drugs are being developed with better outcomes. In order to have a better outcome proper diagnosis along with aggressive therapy and continuous monitoring of patient to assess disease activity and remission but then this becomes a socioeconomic burden. The current treatment options, available, do not fulfill the needs as they provide partial treatment and are required to be used continuously due to remission furthermore there is uncertainty for prognosis of the disorder due to inconsistent and unreliable biomarkers. The high rate of mortality and morbidity is attributed to the systemic complications associated with RA, of which cardiovascular complications are most challenging for patients. Up till now the molecular remission and immunological tolerance in patients remains a mystery to be resolved.

RA is delineated by a hyperplasic synovial cavity, osteoclastogenesis, RF, ACPA and changes in the chemokine and cytokine milieu in synovium accompanied by systemic complications including pulmonary, psychological, cardiovascular and skeletal disorders (McInnes & Schett, 2011). This systemic autoimmune disorder, at initial stages, affects joints such as knee, hands, shoulder and hip joints. The synovial cavity gets inflamed and pannus is formed that destroys the articular structures. Plasma cells, lymphocytes, immune complexes, macrophages and dendritic cells penetrate into the synovial cavity and result in inflammation. In RA macrophages and FLS lead to the membrane expansion thus pannus formation and distuption of articular structure. The cells present in synovuim aggravate the condition by secretion of chemokines, cytokines, MMPs, adhesion molecules, aggrecanases and inhibitors of

metallpproteins that destroy the extracellular matrix thereby impairing and devastating the structure (Bradfield *et al.*, 2003; Firestein, 2003).

2.2 Key Mediators in RA

2.2.1 Adaptive Immune System Activation

Early pathogenesis of RA is attributed to genetics and autoantibodies present in the serum. Cell migration into the synovium starts in the clinical phase of RA after endothelial cells activation that express cell adhesion molecules like selectins, chemokines and integrins. The cytokine production and hypoxia induced stimulate lymphangiogenesis and neoangiogenesis that ultimately results in increased cellular infiltration and establish synovitis. The inflammatory synovial membrane in RA is endorsed to the alterations in micro milieu of synovium and FLS activation (Bouta *et al.*, 2015; Elshabrawy *et al.*, 2015). The architecture of RA synovium is arbitrated by T-helper (Th) cells especially Th1 but studies have shown that Th17 is more profound in RA as it produces TNF-α, IL-17, IL-21, IL-17F and IL-22 that are actively involved in establishment of inflammation (Miossec *et al.*, 2009; Quesniaux *et al.*, 2012) yet the exact role of T cells is to be explored.

Myeloid cells such as macrophages, dendritic cells, basophils, eosinophils, neutrophils and erythrocytes produce cytokines, MHC II molecules and co-stimulatory molecules that facilitate T cell activation and presentation (Lebre *et al.*, 2008; Panayi, 2006). Macrophages and dendritic cells aid in Th17 differentiation and halt regulatory T (Treg) cells activity through Tumor Growth Factor (TGF) β, IL-6, IL-1β, IL-21, IL-23 and TNF-α their synergistic effect is being evaluated (Genovese *et al.*, 2014). Forkhead box P3 (Foxp3) is a key regulator (as transcription factor) in the development, maturation and function of Treg cells that are imbalanced in RA through the action of TNF-α (Alunno *et al.*, 2015). The nonspecific T-cell mediated activation through CD40 & CD40L, ICAM-1 & LFA-1 and CD200 &CD200L serves as an alternative pathway for macrophage and fibroblast activation (Behrens *et al.*, 2007; Noack & Miossec, 2014a, 2014b).

Fundamental role is played by humoral response in RA development. B cells are either present as tertiary lymphoid follicles or as T-cell-B cell aggregates owing to existence of CC Ligand 21 and CXC Ligand14, proliferation inducing ligand (APRIL) which is also known as CD256 or TNF super family member 13 (TNFSF13) and B-Lymphocyte stimulator (BLyS) (Ohata *et al.*, 2005). Plasma cells are present in juxta-articular bone marrow and synovium and play their pathogenic role. Study shows that CD20+ B cells have pathogenic role by the use of rituximab. Although anti-CD20 antibodies do not affect plasma cells through change in autoantibody level but they instigate auto-antigen presentation along with cytokine production (Edwards *et al.*, 2004).

2.2.2 Innate Immune System Activation

Innate immune cells such as mast cells, macrophages and Natural Killer cells (NK cells) present in the synovial membrane are responsible for inflammation. Macrophages are activated by Toll-like receptors (TLRs), immune complexes, cytokines, lipoprotein particles and nucleotide-binding oligomeriation domain (NOD) like receptors and are considered as main culprit because either they release or instigate the production of TNF-α, IL-6, IL-1, IL-23, ROS, NOS, MMPs and prostanoids (Haringman *et al.*, 2005). Studies show that neutrophil extracellular trap (NET) formation plays vital role in maintenance of inflammation in RA (Khandpur *et al.*, 2013). Neutrophils form a NET, through DNA histones and proteins, which confines pathogens and degrades them by antimicrobial peptides (Cascao *et al.*, 2010; Fuchs *et al.*, 2007). Spontaneous NET formation accompanied by increased nuclear translocation of PAD4, ROS, myeloperoxidase (MPO), PAD4 mediated H3 citrullination and neutrophil elastase has been observed in RA (Leshner *et al.*, 2012; Papayannopoulos *et al.*, 2010; Remijsen *et al.*, 2011). These components then for immune complexes with ACPA autoantibodies and trigger further response.

TLRs are upregulated in endothelial cells, FLS, myloid cells, T cells and osteoclast precursors thereby contributing to bone erosion and synovial inflammation through the action of inflammatory proanigogenic factors, cytokines and chemokines (Elshabrawy *et al.*, 2017; Goh & Midwood, 2011; Tamaki *et al.*, 2011). These factors

assist in Th17 polarization and cell recruitment to the inflamed area where osteoclast differentiation occurs. TLR 4 and 5 have been observed to be involved in osteoclast survival and migration of momocytes along with TNF secretion in inflamed area (Chamberlain *et al.*, 2012; Marriott, 2013). Thus TLR involvement increases bone erosion along with inflammation of joint.

2.2.3 Inflammatory Cytokines and Chemokines

At present cytokines and chemokines are arbitrated as most important group of proteins that regulate immune system. Any disruption or dysregulation, due to extrinsic or intrinsic factors, in their expression and mode of action leads to severe imbalance in normal homeostatic functions of immune system and may lead to acute or chronic diseases (Schett & Gravallese, 2012). During initial stages of RA, a distinct profile of cytokines including IL-13, IL-14 and IL-15 stems from T cells that ultimately leads to chronic stage disorder (Raza *et al.*, 2005).

TNF-α acts as a potent regulator of pro-inflammatory cytokines and lead to imbalance of pro-inflammatory and anti-inflammatory cytokines, adhesion molecules expression, stimulation of angiogenesis and suppression of Treg cells thereby disrupting the normal physiological process (Brennan & McInnes, 2008; Hess *et al.*, 2011; Thalayasingam & Isaacs, 2011). IL-6 through autoantibody production and leukocyte activation generate acute phase responses such as anemia, lipid metabolism and cognitive dysfunction. IL-1 family cytokines, IL-1α, IL-1β, IL-18 and IL-33, are also responsible for osteoclast, endothelial cells, chondrocytes and leucocyte activation. IL-17, IL-17 receptor, BLyS, GM-CSF and APRIL are under study as they contribute to the prognosis and complication associated with RA (Auger & Roudier, 1997).

2.2.4 Oxidative Stress

Prognosis of ailments such as RA, diabetes, asthma, allergy, cancer and others is attributed to the oxidative stress generated by reactive oxidative species (ROS). Oxidative stress can be produced due to any factor like trauma, stress, immune system and hormonal imbalance (Cofta *et al.*, 2008). ROS can either be a free radical such as

superoxide, hyroxal, etc or a non-radical like hydrogen peroxide or singlet oxygen, these are formed as a result of intracellular and extracellular activities and then interfere with various cellular processes including cell death (Sena & Chandel, 2012). ROS is produced by cellular sources such as mitochondria as a by-product, it was considered as a defense molecule by neutrophils due to its role as a secondary messenger (Cofta *et al.*, 2008; Phull *et al.*, 2017). In RA the antioxidant system is altered in way that it contributes to ligament degradation through the presence of oxidized IgG, modified low density lipoprotein (LDL), lipid peroxidation products derived from cartilage and nitrous collagen type II in serum, urine and synovial fluid of RA patients (Kaur & Halliwell, 1994; Mateen *et al.*, 2016; Spreng *et al.*, 2001; Uesugi *et al.*, 2000).

2.2.5 Hypoxia

Despite of high synovial vascularity, hypoxia is associated with RA pathogenesis as pO_2 levels drop to 0.46% this is in inverse relation with the inflammatory markers (Harty *et al.*, 2012; Kennedy *et al.*, 2010; Ng *et al.*, 2010). Due to synovial survival and invasiveness there is increased demand of energy that compels the metabolic shift to glycolysis thus glucose level diminish and lactate levels increase along with elevated activity of glycolytic enzymes and mitochondrial dysfunction that leads to abnormal angiogenesis, cellular infiltration and pannus formation (Biniecka *et al.*, 2016; Chang & Chao, 2011; Fearon *et al.*, 2016; Henderson *et al.*, 1979). Mitochondrial components such as mitochondrial DNA (mtDNA) are prone to oxidative damage and play crucial role in processes like apoptosis (Frantz & Wipf, 2010).

2.3 Molecular Pathways in RA

2.3.1 Hypoxia Inducible Factor Signaling

Hypoxia inducible factors (HIF) are expressed in FLS and have been observed as potent regulator of inflammation (Eltzschig & Carmeliet, 2011). Knockouts of HIF-1α and HIF-2α have shown decreased synovial inflammation, cartilage degradation and pannus formation (Cramer *et al.*, 2003; G.-q. Li *et al.*, 2013). HIF

isoforms have been known to up-regulate the expression of inflammatory cytokines like TNF α, IL-6, stromal cell-derived factor 1 (SDF-1/CXCL12), IL-18, IL-15, vascular cell adhesion molecule 1(VCAM-1) and thrombospondin 1 (TSP-1) (Hu *et al.,* 2016). HIF-1α dependent pathway is triggered through hypoxia and TLR-4 expression that in turn increase the expression of COX-2 and IL-6 that contribute to angiogenesis, cellular proliferation and glucose metabolism (Brouwer *et al.,* 2009; Hardy *et al.,* 2014; Hollander *et al.,* 2001; S. Y. Kim *et al.,* 2010). MMPs have been observed to be regulated by HIF expression and hypoxia, but it has been not attenuated by knockdown of HIF-1α (Ahn *et al.,* 2008).

2.3.2 Nuclear Factor-κB Signalling

Nuclear factor κB (NF-κB) pathway is a potent regulator of inflammatory conditions. NF-κB instigates signaling through canonical and non-canonical pathway (also known as classical and alternative pathway) (Basu *et al.,* 1998). NF-κB binds to DNA motifs that in turn instigates the expression of genes involved in cellular development, survival and proliferation along with inflammatory responses (Q. Li & Verma, 2002). Along with the regulation of inflammatory processes NF-κB is also involved in apoptosis, the translocation of NF-κB to nucleus leads to the increased cellular levels of anti-apoptotic proteins that induce angiogenesis (IL-6, MMPs, VEGF and MCP-1), cellular invasion and proliferation (CDKs, cyclins. VCAM-1, ICAM-1 and other proteins) (Escarcega *et al.,* 2007; Prasad *et al.,* 2010). The constitutive activation of NF-κB modifies the microenvironment in a way that pro-inflammatory cytokines and factors prevail and ultimately lead to establishment of chronic inflammation (Berraondo *et al.,* 2016; Dep Prete *et al.,* 2011).

2.3.3 JAK-STAT

Janus kinase and signal transduction and activation of transcription (JAK-STAT) is associated with the increased production of inflammatory cytokines such as IL-6 in RA against which inhibitors like Tofcitinib are used as treatment options (Fearon *et al.,* 2016; Oliver *et al.,* 2009). Transcription of pro-inflammatory genes sets off after the phosphorylation of JAK and forms a homodimer that then phosphorylates STAT (Darnell *et al.,* 1994). Tofacitinib primarily affects the

production of IL-2, IL-15, IL-4, IL-21, IL-6, IL-23, IL-12 and INF-γ that then lead to Th1, Th2 and Th17 differentiation (Ghoreschi *et al.*, 2011). Combinatory therapy of tofacitinib and methotrexate has been proven to be efficacious in RA patients during clinical trials by reducing the arthritic symptoms (Burmester *et al.*, 2013; Y. H. Lee *et al.*, 2015).

2.4 Apoptosis and Autophagy Interplay

Many apoptosis regulating proteins induce autophagy, as BH3 has been observed to interrupt Beclin-1 and Bcl-2 interaction that promotes autophagy, thereby implying that these two processes are closely linked to each other. The mode of action of p53, a tumor suppressor gene, varies with its location in the cell as intrinsic pathway is triggered by modulation of the pro-apoptotic proteins like Bax, Bid, p53 upregulated modulator of apoptosis (PUMA) and Noxa that induce release of cytochrome c from mitochondria, when p53 is present in cytoplasm. Extrinsic pathway is triggered, if p53 is in nucleus, through Fas and TRAIL (TNF-related apoptosis-inducing ligand) receptor expression. Moreover genotoxic stress initiated by nuclear p53 induce damage-regulated autophagy modulator (DRAM) to promote the auto-lysosome formation, whereas cytoplasmic p53 represses autophagy via mTOR signaling. The p53-inducible BH3-only protein, PUMA triggers mitochondrial degradation depending on Bax/Bak and lead to apoptosis. This signaling cascade does not interfere with Beclin-1 and Bcl2/BclxL. For better understanding of the process other proteins and transcription factors should be explored (Gordy & He, 2012; Mukhopadhyay *et al.*, 2014).

Autophagy and apoptosis are types of cell death, essential for controlling a lot of important mechanisms in the body. However if processes are under or overexpressed in cells then this might turn out to be fatal for the cells causing various anomalies or onset of different diseases as in case of RA in which apoptosis and autophagy balance is disturbed. Inhibitors of apoptosis proteins (IAPs) regulate apoptosis by binding to caspase-3, caspase-9, caspase-8, caspase-10 and caspase-9 in normal state these caspases in response to proapoptotic signals compel mitochondria to release cytochrome c and lead cell to death by blocking IAPs (Deveraux *et al.*,

1998; Du *et al.*, 2000; Lattuada *et al.*, 2015; Verhagen *et al.*, 2000). These IAPs are closely linked to RA as they are one of the key factors that generate apoptosis resistance in FLS and promoting cell survival via autophagy (Cuda *et al.*, 2016; Gordy *et al.*, 2011). Synovial hyperplasia is mainly due to reduced apoptosis, this resistance to apoptosis achieved by FLS is mainly due to increased level of anti-apoptotic factors, autophagy and down regulation of pro-apoptotic factors (Huber *et al.*, 2006; Kato *et al.*, 2014; Shin *et al.*, 2010; Xu *et al.*, 2015; Xu *et al.*, 2013).

2.5 Apoptosis and Autophagy Modulation

Although multiple factors contribute to the prognosis and pathogenesis of rheumatoid arthritis but apoptosis and autophagy imbalance contributes to the further complications of the disorder as a relationship between cell survival and mitochondrial dysfunction in FLS has been established moreover Th17 and IL-17 have been observed to be involved in the disturbance of the apoptosis-autophagy homeostasis (E. K. Kim *et al.*, 2017). Inhibition of autophagy in animal models have shown to attenuate synovial inflammation and hyperplasia thereby promoting apoptosis via PI3K/AKT pathway (S. Li *et al.*, 2017). Another also confirmed the results by suggesting that hypoxia contributes to the autophagy induction and proliferation of FLS, whereas if autophagy inhibitors are administered then the inflammatory process is reduced (Yang *et al.*, 2017). There is possibility that increased apoptosis may be beneficial or may interfere with autophagy and culminate the inflammatory process. Studies have shown that apoptosis induction in mice models lead to amelioration of inflammation and pannus growth in RA (Kawahito *et al.*, 2000). Modulation of autophagy and apoptosis can be explored as a treatment option as both processes can be halted at any step of the process.

2.6 Ethno-Medicine for RA

Plant species that belong to family Asteraceae, Solanaceae, Fabaceae, Leguminoseae, Brassicaceae, Ranunculaceae, Zygophyllaceae, Caparaceae, Polygonaceae, Ephedraceae, Lamiaceae, Euphorbiaceae, Betulaceae and Poaceae have been used against RA (Kamal *et al.*, 2016). Members of family Asteraceae and Solanaceae have been found effective against RA may be due to high flavonoid,

steroid and alkaloid content (Abad *et al.*, 2012; Shravan *et al.*, 2011). Ethnobotanically various plant parts are used against various diseases such as RA (Table 2.1).

Table 2.1: Summary of ethnobotanically important plants from different areas of Pakistan used for treatment of RA.

Botanical Name	Family	Local Name	Location	Part Used	Reference
Mentha longifolia	Lamiaceae	Welana	Bannu, Upper Dir	Powdered dry leaves	(Hazrat *et al.*, 2011)
Mentha sylvestris	Lamiaceae	Welana	Gilgit, Waziristan, Brimal	Leaves and flowers	(Ullah *et al.*, 2013)
Moringa oleifera	Moringaceae	Suhanjhna	Khushab, Noorpur Thal	Fruit and flowers	(R. Qureshi *et al.*, 2011)
Justica adhatoda	Acanthaceae	Aursa Baza Baikar	Darra Adamkhel Valley, Kohistan, Batkhela	Roots, Bark, leaves and flowers	(Adnan *et al.*, 2014)
Lactuca sativa	Asteraceae	Dodal	Siran Valley, Mansehra	Roots	(Mahmood *et al.*, 2011)
Juglans regia	Juglandaceae	Not known	Karakorum region	Stem	(Sheikh *et al.*, 2002)
Cassia fistula	Fabaceae	Amaltas	Islamabad-Lahore moterway	Roots, leaves, flowers and seeds	(Adnan *et al.*, 2014)
Centella asiatica	Leguminosae	Not known	Bannu	Leaves	(Kamal *et al.*, 2016)
Carthamus oxyacantha	Asteraceae	Kunzala	Bannu	Seed oil	(Kamal *et al.*, 2016)
Acacia modesta	Mimosaceae	Phulai Reenn	Mianwali, Isakhel,	Leaves, bark, wood	(Adnan *et al.*, 2014)

			Barroha	and gum	
Acacia nilotica	Mimosaceae	Kikar	Cholistan	Seeds	(Adnan *et al.*, 2014)
Achyranthes aspera	Amaranthaceae	Phut kanda Lainda	Siran valley, Dera Ghazi Khan, Nara Dessert	Roots, stem, fruit, flower and leaves	(Mahmood *et al.*, 2011)
Aloe vera	Xanthorroeace	Kanwargandal Aloe	Attock, Jalalpur Jatan, Gujrat, Punjab	Leaves	(Hayat *et al.*, 2008)
Brassica campestris	Brassicaceae	Sarson	Dera Ghazi Khan	Leaves, seeds and flowers	(R. Qureshi & Bhatti, 2009)
Brassica nigra	Brassicaceae	Kali sarson	Mianwali, Isakhel	Whole plant	(Mahmood *et al.*, 2011)
Hyoscyamus niger	Solanaceae	Khoob Kalan	Dera Ghazi Khan	Whole Plant	(R. Qureshi & Bhatti, 2009)
Salix alba	Salicaceae	Baid e majno	Bhumbart valley, Chitral	Whole plant	(Ali & Qaiser, 2009)
Eruca sativa	Brassicaceae	Tare mere	Isakhel, Makarwal and Gulla Khel	Leaves and seeds	(Mahmood *et al.*, 2011)
Brassica rapa	Brassicaceae	Turnip	Karachi	Leaves and roots	(Alam *et al.*, 2011)
Buxus papillosa	Buxaceae	Pepper	South Punjab	Whole plant	(R. Qureshi & Bhatti, 2009)

2.7 *Thymus serpyllum*

Thymus serpyllum of family Lamiaceae is a native plant of Himalayan range with significant ethnobotanical importance in Gilgit Baltistan. Traditionally it is used to alleviate the symptoms of gastrointestinal problems, fever, respiratory problems and menstrual issues (Jabeen *et al.*, 2015).

2.7.1 Ethno-botany of *Thymus serpyllum*

Thymus serpyllum is locally used for cold, bronchitis, fever, cough, respiratory and gastrointestinal problems and toothache (Jabeen *et al.*, 2015). Infusions and tonic prepared using aerial parts of the plant have been manipulated to cure liver, stomach, kidney and eye problems along with blood purification (Jabeen *et al.*, 2015). Plant is widespread and used for the treatment of different diseases and conditions (Table 2.2).

Table 2.2: Ethnobotanical importance of *Thymus serpyllum* in various regions of world especially Pakistan.

Disease Cure	Country Region	Traditional recipe	Reference
Anthelmintic	Europe	Tonic prepared using aerial parts	(Quave *et al.*, 2012)
Rheumatism	Alpine region of Italy	Infusion prepared from aerial parts	(Cappelletti *et al.*, 1982)
Sedative	Western Balkans	Infusion prepared from aerial parts	(Mustafa *et al.*, 2012; S Redžić, 2007)
Menstrual Disorders	India	Tonic prepared using aerial parts	(Gairola *et al.*, 2014)
Eczema	Pakistan	Tea or infusion prepared	(Benítez *et al.*, 2010)
Carminative	Europe	Tonic prepared using aerial parts	(Chevallier, 1996; Quave *et al.*, 2012)
Anti-cholestrolemic	Western Balkans	Infusion prepared from aerial parts	(Mustafa *et al.*, 2015)
Reduce swelling	Pakistan	Tea or infusion prepared	(Kozuharova *et al.*, 2013)
Flu and fever	Gilgit Baltistan Pakistan	Tea from aerial parts of plant	(Jabeen *et al.*, 2015)
Bronchitis	Bagh Azad Kashmir Pakistan	Tea from aerial parts of plant	(R. Qureshi *et al.*, 2011)

Gastrointestinal problems	Gilgit Baltistan Pakistan	Tea from aerial parts of plant	(Jabeen *et al.*, 2015)
Sedative	Europe	Tonic prepared using aerial parts	(Chevallier, 1996; Quave *et al.*, 2012)
Immuno-stimulant	Western Balkans	Infusion prepared from aerial parts	(Mustafa *et al.*, 2015)
Treating wounds	Pakistan	Tea or infusion prepared	(Mati & de Boer, 2011)
Anti-diarrheal	Catalonia and Balearic Islands	Infusion prepared using whole plant	(Carrió *et al.*, 2012)
Respiratory Problems	Gilgit Baltistan Pakistan	Tea from aerial parts of plant	(Jabeen *et al.*, 2015)
Disinfectant	Europe	Tonic prepared using aerial parts	(Chevallier, 1996; Quave *et al.*, 2012)
Blood Circulation	Western Balkans	Infusion prepared from aerial parts	(Mustafa *et al.*, 2015)
Liver and Kidney problems	Bagh Azad Kashmir Pakistan	Tea from aerial parts of plant	(R. A. Qureshi *et al.*, 2007)
Laxative	Bagh Azad Kashmir Pakistan	Tea from aerial parts of plant	(R. A. Qureshi *et al.*, 2007)
Blood purification	Bagh Azad Kashmir Pakistan	Tea from aerial parts of plant	(R. A. Qureshi *et al.*, 2007)
Antispasmodic	Europe	Tonic prepared using aerial parts	(Chevallier, 1996; Quave *et al.*, 2012)
Antiseptic	Europe	Tonic prepared using aerial parts	(Chevallier, 1996; Quave *et al.*, 2012)

2.7.2 Ethno-pharmacology of *Thymus serpyllum*

Extracts of *Thymus serpyllum* have been exploited to explore its potential in various conditions (Table 2.3).

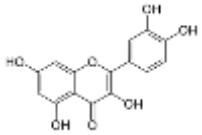
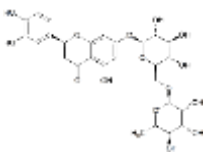
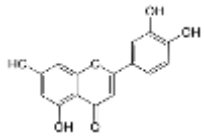
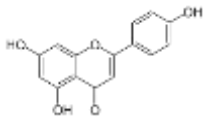
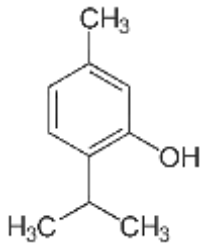
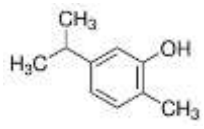
Table 2.3: Summary of ethnopharmacological properties of *Thymus serpyllum* extracts prepared in different solvents.

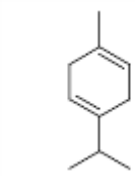
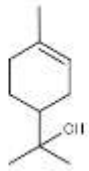
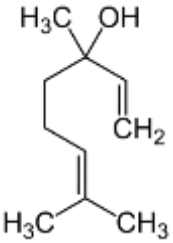
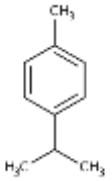
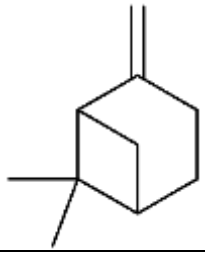
Extract	Pharmacological Activity	Assay Used	Reference
Ethanol	Antioxidant	Hydroxal Radical Scavenging Method	(Joshi & Juyal, 2018)
Ethanol	Antibacterial	Antibacterial assay	(Abramovic *et al.*, 2018)
Aqueous-ethanol	Anti-tumor	Cell culture and animal model	(Aralbaeva *et al.*, 2017)
Aqueous-ethanol	Cytotoxicity	Cell culture	(Aralbaeva *et al.*, 2017)
Aqueous	Anti-diabetic	Diabetic mice model	(Mushtaq, 2017)
Ether	Anti-diabetic	Diabetic mice model	(Mushtaq *et al.*, 2016)
Aqueous-ethanol	Hepatoprotective	CCL_4 induced liver toxicity	(Aralbaeva *et al.*, 2017)
Aqueous	Anti-diabetic	Diabetic mice model	(Mushtaq *et al.*, 2016)
Aqueous	Anti-hypertensive	Animal Model	(Mihailovic-Stanojevic *et al.*, 2013)
Aqueous-ethanol	Lipid peroxidation	Animal model	(Aralbaeva *et al.*, 2017)
Aqueous	Anti-inflammatory	Colitis mouse model	(Algieri *et al.*, 2014)
Methanol	Epigenetic modification	Acetone deacetylase activity assay	(Bozkurt *et al.*, 2012)
Methanol	Anti-cancer	Cell line evaluation	(Bozkurt *et al.*, 2012)
Methanol	Cytotoxicity	Cell line evaluation	(Bozkurt *et al.*, 2012)
Hexane	Anti-tumor	Cancer cell line tests	(Baig *et al.*)
Aqueous	Anti-microbial	Anti-bacterial Assay	(Ultee *et al.*, 2002)

2.7.3 Biological Active Compounds of *Thymus serpyllum*

The biologically active compounds isolated from *Thymus serpyllum* have not been characterized yet however some of the essential oils are being explored for their therapeutic potential (Table 2.4).

Table 2.4: Summary of bioactive compounds identified in *Thymus serpyllum* extracts prepared in different solvents and possess pharmacological properties.

Chemical Class	Compound	Structure	Molecular Formula	Molecular Weight(g/mol)	Reference
Flavonoids	Quercetin		$C_{15}H_{10}O_7$	302.23	(Kulišić *et al.*, 2007)
	Eriocitrin		$C_{27}H_{32}O_{15}$	596.54	
	Luteolin		$C_{15}H_{10}O_6$	286.2	
	Apigenin		$C_{15}H_{10}O_5$	270.2	
Terpenoids	Thymol		$C_{10}H_{14}O$	150.22	(Jarić *et al.*, 2015)
	Carvacrol		$C_{10}H_{14}O$	150.2	

	γ Terpenine		$C_{10}H_{16}$	136.2	
	α Terpineol		$C_{10}H_{18}O$	154.2	
Polyphenols	Linalool		$C_{10}H_{18}O$	154.2	(Jarić *et al.*, 2015)
	p-cymene		$C_{10}H_{14}$	134.2	
	β pinene		$C_{10}H_{16}$	136.2	

2.7.4 Pharmacological Activities of Bioactive Compounds from *Thymus serpyllum*

The phytochemical compounds isolated from plant sources have less side effects and have been proven to be more efficacious. Table 2.5 comprises of the list of pharmacological activities of the bio active compounds isolated or studied up till now.

Table 2.5: Bioactive compounds identified in *Thymus serpyllum* extracts and essential oils and their pharmacological properties.

Compound	Pharmacological activity	Reference
Thymol	Antiseptic Anti-cancerous Anti-proliferative Anti-microbial	(Deb *et al.*, 2011; Nikolić *et al.*, 2014)
Carvacrol	Anti-cancerous Anti-bacterial	(Gedara, 2008; Jaafari *et al.*, 2007)
p-cymene	Anti-bacterial	(Ultee *et al.*, 2002)

2.8 *Thymus serpyllum* and Disease Conditions

Thymus serpyllum has been exploited in order to have an insight about disease management, treatment efficacy and bioactive components involved. Plant has not been explored to fullest and there be need for further characterization and evaluation.

2.8.1 Anti-microbial Activity

Anti-microbial assays revealed that ethanol and aqueous extracts of *Thymus serpyllum* have strong bactericidal activity owing to the presence of carvacrol and p-cymene that synergistically elicit inhibitory activity against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Vibrio cholera, Salmonella typhi, Lactobacillus acidophilus, Bacillus megaterium, Staphylococcus albus, Shigella ferarie, Micrococcus luteus* and *Bacillus subtilis* (Ahmad *et al.*, 2006; Ultee *et al.*, 2002). The polyphenolic compounds present in essential oils of *Thymus serpyllum* ameliorated mycotoxin and growth of fungal species like *Aspergillus ochraceus, A. niger* and *A. carbonarius* (Sokolić-Mihalak *et al.*, 2012).

2.8.2 Anti-tumor Activity

Anti-tumorigenic activities *Thymus serpyllum* extracts have been reported such as the hexane extract demonstrated cytotoxic effect on liver carcinoma (HepG2), Lung carcinoma (A549), breast cancer (MCF-7 and MDA-MB-231), colon cancer (HCT-116) and prostate cancer (PC3) cell lines (Baig *et al.*). Essential oils of *Thymus serpyllum* consist of thymol and carvacrol as most abundant components. Carvacrol demonstrated cytotoxic effects leukemia and liver carcinoma (Gedara, 2008; Jaafari *et al.*, 2007). Whereas thymol exhibited strong anti-proliferative and anti-cancer properties (Deb *et al.*, 2011; Nikolić *et al.*, 2014).

2.9 *Thymus serpyllum* and Apoptosis

Methanolic extract of *Thymus serpyllum* of has shown pro-apoptotic activity by increasing caspase 3 and caspase 7 expression and activity in MDA-MB-231 (breast cancer cell line) (Bozkurt *et al.*, 2012).

CHAPTER 3 : MATERIALS AND METHODS

3.1 Plant Material Collection

3.1.1 Plant Material Collection and Storage

Plant sample was collected from Rakaposhi Base camp, Nagar valley, Gilgit Baltistan. Plant material was dried and stored at room temperature till further processing.

3.1.2 Herbarium Preparation

Whole plant with inflorescence was cleaned and dried under heavy weight for few days and placed on herbarium sheet for submission to Pakistan Museum of Natural History for obtaining voucher number.

3.2 Plant Barcoding

3.2.1. DNA Extraction

Plant DNA was isolated through modified version of Dolye and Doyle plant DNA extraction protocol (Doyle, 1991). 1g of plant material was taken and ground in liquid nitrogen until fine powder was made to which pre-heated at 60 ˚C CTAB was added and the slurry was transferred to falcon tube and placed in shaking incubator at 60˚C. To the slurry mixture of phenol-chloroform and isoamylalcohol was added of equal volume and mixture was divided into two falcons and centrifuged at 9000rpm for 10 minutes at room temperature. The aqueous phase was transferred to another falcon and ice cold isopropanol (Merck) was added and incubated overnight. Centrifugation was done at 9000 rpm for 10 minutes and supernatant was discarded and 1 ml of wash buffer was added, gently agitated and incubated for 20 minutes after which centrifugation at 9000 rpm for 5 minutes and the supernatant was discarded and pallet was allowed to dry for 30 minutes at 37˚C. 1 ml TE buffer was added and stored at -20˚C until further processing.

3.2.2. Agarose Gel Electrophoresis for DNA

DNA was qualitatively analyzed by 1% agarose gel electrophoresis. 5 µl of DNA sample was mixed with 2 µl of 6X loading buffer and loaded into wells along with 100 bp gene ruler (Thermofisher Scientific) at electrical potential of 60 Volt. Observations were made at Ultraviolet Transilluminator (Wealtech).

3.2.3. NanoDrop for DNA

DNA was quantified using Thermo Scientific NanoDrop ND-2000 and Proceeded for PCR amplification of conserved regions.

3.2.4. PCR for Conserved Region Amplification

Ribulose bisphospahte carboxylase Large region (rbcL) was amplified using the program shown in Figure 3.1. 1 µl DMSO, 1 µl BSA, 2.5 µl 10X Taq Buffer, 2.5 µl 2mM dNTPs, 1.5 µl 50mM $MgCl_2$, 0.5 µl Forward and Reverse primers (Table 3.6) were mixed with 0.25 µl *Taq Polymerase* and 1 µl DNA (Table 3.1) and were placed in thermocyler at conditions given in Figure 3.1.

Table 3.1: Recipe for rbcL amplification

Component	**Quantity(µl)**
10X Taq Buffer	2.5 µl
2mM dNTPs	2.5 µl
50mM $MgCl_2$	1.5 µl
20mM Forward Primer	0.5 µl
20mM Reverse Primer	0.5 µl
Taq Polymerase	0.25 µl
Bovine Serum Albumin (BSA)	1 µl
Dimethylsulphoxide (DMSO)	1 µl
Nuclease Free Water	14.3 µl
DNA	1 µl
Total Volume	25 µl

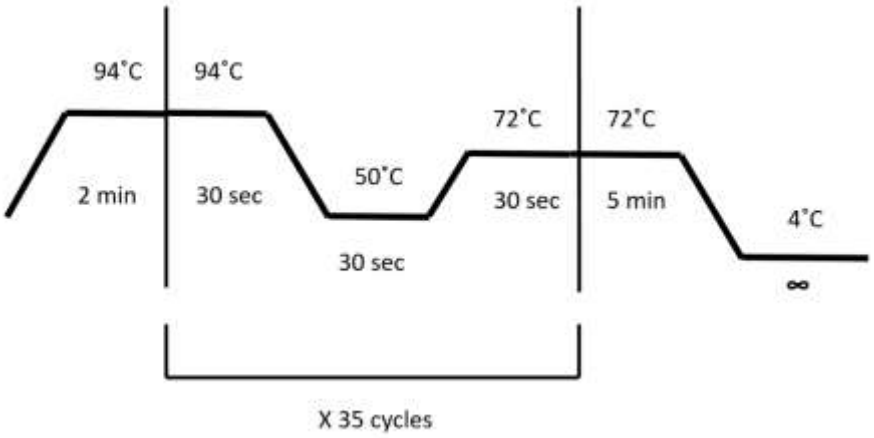


Figure 3.1: PCR Program for rbcL

3.2.5. Agarose Gel Electrophoresis for Amplicon

2% agarose gel was prepared heating 2g of agarose and 120ml of 1X TBE buffer in microwave, after which 3 µl of ethiduim bromide was added and allowed to set. 3 µl of 50 bp gene ruler along PCR amplicons were loaded into the wells and 60 volts were provided for 45 minutes after which gel was analyzed on Ultraviolet Transilluminator (Wealtech).

3.2.6 Sequencing

Amplified PRC product was treated with *exosap IT*, 2 µl in 5 µl of amplicon and incubated. For sequencing samples were sent to Macrogen.

3.2.7 Phylogenetic Analysis

Plant sequence analysis was done through Maximum Likelihood (ML) method and a phylogenetic tree was plotted.

3.3 Plant Extract Preparation

3.3.1. Maceration

Plant material was rinsed with water and then air dried after which it was ground to fine dust powder. Plant material was added to solvent in according to 1:10; 1g of plant material was dissolved in 10 ml of solvent. Water and ethyl acetate were

selected as solvents and extracts were left for four weeks with frequent shaking (three times a day) in a dark place.

3.3.2 Filtration

After four week maceration, the extract was filtered with Watmann filter paper 1 in flask. During process of filtration light and temperature precautions were taken to avoid adulteration of the extract. The filtrate was then shifted to rotary evaporator and debris were discarded.

3.3.3 Rotary Evaporation

Boiling points of solvents were determined from literature and temperature of water bath was set in accordance to that Table 3.2. Filtrate was fixed onto the apparatus and extract was concentrated. At certain concentration the extract was taken off the apparatus and then air dried to obtain a solid extract, which was used for characterization and treatment of the animal model.

Table 3.2 Boiling points of solvents used for extract preparation.

Solvent	**Boiling Point**
Water	100.00°C
Ethyl Acetate	77.6°C

3.4 Plant Extract Characterization

3.4.1. Biochemical Tests

Alkaloids

Hager's Test was used to detect alkaloids. To 2 ml of extract few drops of Hager's reagent (1g of picric acid mixed with 100 ml water) was added and yellow precipitate formation indicated alkaloids.

Phenols

To 1 ml of extract few drops of 1% $FeCl_3$ were poured and bluish black coloration confirmed presence of phenols.

Anthraquinones

Borntrager's Test was performed to detect anthraquinones. 3 ml of extract was mixed with 3 ml of benzene and 5 ml of 10% NH_3 that lead to pink, violet and red coloration as confirmation of anthraquinones.

Flavonoids

1 ml extract was mixed with 1 ml of 10% $Pb(C_2H_3O_2)_4$ (lead acetate) and yellow precipitate formation indicated presence of flavonoid.

Anthocyanins

To 2 ml of extract 2 ml of HCl (2N) was added after which 1 ml of NH_3 was poured and color change of pinkish red to bluish violet indicates anthocyanins.

Leucoanthocyanins

5 ml of extract was mixed with 5 ml of isoamylalcohol and the organic layer turns into red in the presence of leucoanthocyanins.

Tannins

To detect tannins Braymer's Test was done. 2 ml of extract and water were mixed and few drops of 5% $FeCl_3$ were poured into for transient greenish to black color formation.

Phalobatannins

Precipitate Test was done to detect phalobatannins, 2 ml of extract and 1% HCl were mixed and boiled until formation of red precipitates.

Coumarins

2 ml of extract was mixed with 3 ml of 10% NaOH to detect presence of coumarins by yellow color formation.

Terpenoids

To 2 ml of extract and $CHCl_3$ (chloroform) 1 ml of ethanol was added incubated on heat for 2 minutes and 3 drops of concentrated H_2SO_4 were added for deep red coloration for terpenoid presence.

Diterpenes

Few drops of copper acetate were added to the mixture of 2 ml extract and water and formation of emerald green color indicated the presence of diterpenes.

Triterpenes

1 ml of extract was mixed with few drops of concentrated H_2SO_4, shaken and allowed to stand until the formation of yellow color in the lower layer.

Steroids

2 ml of extract, $CHCl_3$ and concentrated H_2SO_4 were mixed to observe a reddish brown coloration at interface as confirmation of steroids.

Sterols

To detect Salkowski's Test was done in which 1 ml of extract was mixed with few drops of concentrated H_2SO_4, shaken and held for red color appearance in the lower layer.

Saponins

Foam Test was done to confirm the presence of saponins. 5 ml water and extract were mixed and heated until froth appeared as indication.

Resins

2 ml of extract was mixed with 3 ml of acetone and HCl and was provided heat in water bath for 30 minutes and a color change from pink to red indicated presence of resins.

Glycosides

Salkowski's Test was done to detect the presence of glycosides. To 2 ml of extract 2 ml of each $CHCL_3$ and concentrated H_2SO_4 were added and reddish brown ring was formed at junction.

Cardiac Glycosides

For detection of cardiac glycosides 2ml of acetic acid along with few drops of $FeCl_3$ and 1 ml of concentrated H_2SO_4 were poured to 2 ml of extract violet to brown coloration confirmed the presence.

Proteins

To detect proteins xanthoproteic test was performed. To 1 ml of extract 1 ml of concentrated H_2SO_4 was added to obtain white precipitate that turn yellow on boiling.

Amino Acids

For this experiment Ninhydrin Test was done. Few drops of Ninhydrin (1g of indane 1, 2, 3 trione hydrate was dissolved in 100 ml n-butanol) were added to 1 ml of extract was obtain, formation of violet color indicated presence of amino acids.

Carbohydrates

Fehling's Test was opted for carbohydrates. 1 ml of Fehling's solution A and B were added to 2 ml of extract and the mixture was heated for a while until red precipitates were formed.

Deoxysugers

To 2 ml of extract 2 ml of acetic acid and 1 ml of concentrated H_2SO_4 along with 1%$FeCl_3$ were added and incubated for some time to obtain result as a brown ring formation.

3.4.2. 2, 2,-Di-Phenyl-2-Picryl Hydrazyl Hydrate (DPPH) Assay

DPPH assay was performed in order to evaluate the free radical scavenging activity of ethyl acetate and aqueous extract using already mentioned protocol (SANGANNA *et al.*) with some modifications. Concentrations of 15µg, 20µg, 30µg, 50µg and 75µg were adopted for extracts and standard ascorbic acid with 0.1mM concentration of DPPH. To 1250µl of 250µl of 0.1mM DPPH was added and incubated for 60 minutes and color change was observed. Optical Density (OD) was measured on spectrophotometer at 517nm.

3.5 Animal Procurement and Model Establishment

3.5.1. Animal Acclimatization

8-12 week old female *BALB/c* mice, weighing 30-35g, were obtained from ASAB Animal House facility. All of the experimental mice models were housed at Animal Laboratory ASAB, NUST. Regulated 16:8 ratio sunlight to darkness was provided for efficient circadian cycles. To evaluate any phenotypic changes F1 progeny was evaluated.

3.5.2. Animals Utilized for Experiment

The experimental procedures and protocols were approved by Institutional Review Board at ASAB (IRB-ASAB). Animals were screened for any physiological and pathological anomalies. All characters such as hair color, tissue damage or any other defect were evaluated beforehand. Standard feed and water provided to mice was treated with Ultraviolet radiation to avoid contamination. All procedures carried out during the course of study were in accordance with guidelines provided by National Institute of Health (NIH).

3.5.3. Arthritis Induction

Arthritis was induced using Bovine Type 2 Collagen and complete Freund's adjuvant. Immunization mix was made using 0.1 M acetic acid (1.15 ml dissolved in 200ml of 0.89% Hartmann's solution) to dissolve bovine type 2 collagen and kept at ice. Collagen type 2 solution and complete Freund's adjuvant were mixed in 1:1 and vortexed for 2-3 minutes after which BSA (1 mg dissolved in 1ml of 0.89% Hartmann's solution) was added in 2:1 concentration and mixed through pipetting. 31 gauge insulin syringes were filled with immunization mix to 0.2ml for injecting the mice in sub-dermal region of tail (Figure 3.2). Caution was taken against puncturing the dorsal and lateral coccygeal veins, which can lead to death. Infusions were given to mice on day 0, 7 and 14. On Day 21 and 28 booster dose of complete Freund's adjuvant were given in hind paws and care was taken not to puncture any vein. Degree of inflammation was taken as marker for induction of arthritis.

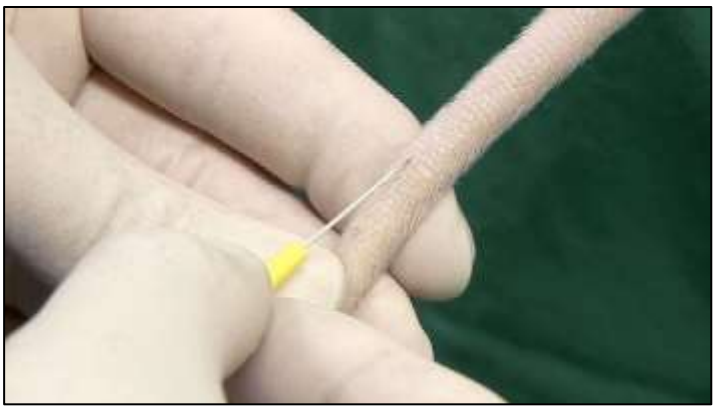


Figure 3.2: subcutaneous injection in tail

3.5.4. Exclusion and Inclusion Criteria

The arthritic severity was elucidated with grading of each paw according to the criteria provided in Table 3.3. Animals that developed 3 or 4 degree arthritis were selected for further experimental procedures.

Table 3.3: Grading of Arthritis

Grade	Condition
0	No swelling and focal redness
1	Swelling of finger joints
2	Mild swelling of ankle, wrist and other joints
3	Severe inflammation of entire paw, angiogenesis and abscessation
4	Severe abscessation, deformity of joints, ankylosis and rheumatic nodules.

3.5.5. Experimental Design

Mice divided into six groups 10 mice/group and were housed in 12 cages 5 mice/cage. 10 cages encased arthritic mice whereas 2 cages held normal mice. Plant extracts were prepared and given to experimental models. Groups are given in Table 3.4.

Table 3.4: Experimental groups used during the course of experiment.

Group	Description of Group	Type of Group
1	660mg/kg Aqueous Extract	Experimental Group
2	660mg/kg Ethyl acetate Extract	Experimental Group
3	10 mg/kg Leflunamide	Positive Control 1
4	25 mg/kg Hydroxychloroquine	Positive Control 2
5	Arthritic group not given treatment	Negative Control
6	Normal mice without arthritis	Normal

3.6 Measurement of Paw and Joint Edema

From day 0, the primary immunization day, hind paws of mice were measured with vernier caliper (GmBH) on daily basis. Three measurements were taken for each paw and mean value was taken. Measurements were taken in the presence of an observer who had no knowledge of the groups.

3.7 Treatment with Extract

Aqueous and ethyl acetate extract at 660mg/kg concentration were given to mice. Standard drugs, Leflunamide and Hydroxychloroquine, were administered at 10 mg/kg and 25 mg/kg respectively. Plant extract and standard drugs were mixed in feed of mice and was given daily for six weeks. On Day 30 (two days after last booster dose), extract administration was started as mice were given a period of fasting.

3.8 Dissection and Organ Collection

Mice were anesthetized using chloroform and blood was collected via transthoracic cardiocentesis (Figure 3.3) at the end of experiment. Mice were euthanized in anesthesia state by cervical dislocation after which hind paws, skeletal muscles and spleens were collected.

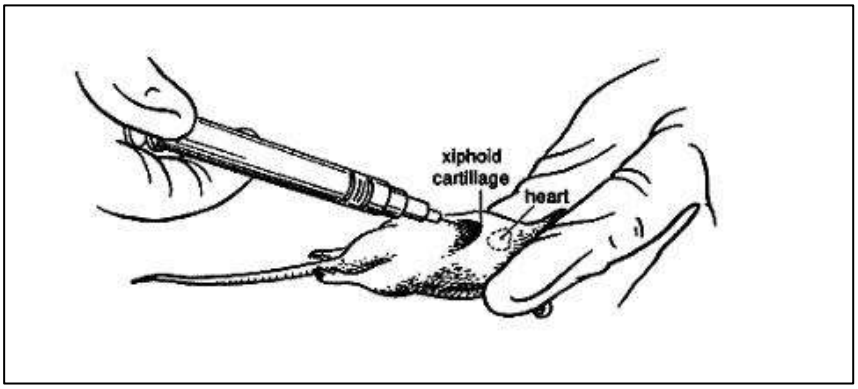


Figure 3.3: Transthoracic cardiocentesis

3.9 Spleen Indexing

Spleen indexing was done as previously described in (R. Huang *et al.*, 2013). Length, width and height of spleen were measured with vernier capllier.

3.10 Histopathology

The resected hind paws were then kept in 10% formalin for 24 hours after which they were subjected to decalcification in 5% nitric acid for 48-72 hours. 70%,

80% and 100% concentrations of ethanol were prepared into which decalcified paw samples were immersed for 2 hours each. Then for 2 hours samples were immersed in 50:50 isopropanol: xylene after which they were immersed in 30:70 isopropanol: xylene for 2 hours. Then for another 2 hour span samples were immersed in 100% xylene after which samples were loaded into cast for pouring of liquid paraffin. After drying samples were proceeded for sectioning through microtome and then the section ribbon was fixed on slide, using hot and cold water bath cycle, for staining. Using hematoxylin and eosin slides were stained after rehydration and then sealed with DPX mountant and coverslip after which they were observed with light microscope at 4X, 10X and 40X to observe changes amongst the study groups.

3.11 Immunohistochemistry

3.11.1 Sample Preparation

Samples stored in 10% formalin were placed in mold that was coated with liquid paraffin and allowed to cool. 4-6 μm sections were diced through a microtome and floated in hot water bath after which sections were mounted on slides. For antibody staining, paraffin was removed and samples were placed in xylene containers 5 minutes each, three containers of xylene were prepared. Samples were placed in 100% ethanol for 10 minutes and then in 95% ethanol for 10 minutes again. Then samples were placed in dH_2O for 5 minutes.

3.11.2 Primary Antibody Labelling

Antibody dilutions were made following the manufacturer's instructions. Sample was pre-incubated for 10 minutes in 5% BSA and slides were drained and 100μl of primary antibody, LC3b was poured over the slide and incubated for 60 minutes at 37˚C. After which slides were rinsed with PBS and placed in wash bath for 5 minutes.

3.11.3 Secondary Antibody Labelling

Secondary antibody, peroxidase or alkaline phosphatase were diluted according to manufacturer's instructions and 100μl of it was poured over the slide after the removal of PBS and then slides were incubated at 37˚C for 30 minutes. Slides were washed with PBS for 5 minutes.

3.11.4 Antibody Substrate Treatment

Excess PBS was removed from slides and few drops of substrate were poured over antibody labelled slides and incubated for 5-10 minutes that allowed enough reaction time and the reaction was terminated using distilled water.

3.11.5 Counterstaining

Mayer's hematoxylin was applied on slide and incubated for 0.5-5 minutes and rinsed with distilled water and final glycerol gelatin was used as mountant and then slides were sealed with coverslips.

3.11.6 Microscopy

Slides were observed under microscope at 4X, 10X, 40X and 100X for final cell counting and slide grading.

3.12 Real-Time PCR

3.12.1 RNA Extraction

RNA isolation was done from whole blood using TRIZOL method (Simms *et al.*, 1993). 750 µl of TRIZOL Reagent and 20 µl of 5N Glacial Acetate were added to 200 µl of whole blood, in an eppendrauf tube, and was mixed vigorously for 15 seconds followed by 5 minute incubation on ice. The mixture was supplemented with 200 µl of Chloroform (Sigma-Aldrich) and vigorously shaken for 15 seconds and incubated for 2-5 minutes on ice. Sample was centrifuged at 12,000g at 4°C for 15 minutes after which phase separation was done; the aqueous phase was transferred to a new tube to which 500 µl of isopropanol (AnalaR) was added and then incubated for 5-10 minutes on -20˚C. For 8 minutes at 4°C sample was centrifuged at 14,000g after which supernatant was discarded and 1 ml of 75% ethanol was added and 5 minute centrifugation at 4°C at 12,000g was done. After which sample was air-dried and dissolved in 25 µl of DEPC-treated water and stored at -80˚C.

3.12.2. Gel Electrophoresis

Qualitative analysis of RNA was done by 1% gel electrophoresis (0.7g agarose, 70ml 1X TBE Buffer, 3 µl of ethidium bromide). 5 µl of RNA was combined

with 2 µl of 6X loading dye and 100 bp gene ruler were loaded in wells and 60 volts for 90 minutes were provide. Results were analyzed on Dolphin Doc (Wealtech).

3.12.3. RNA Quantification

RNA was quantified using Thermo Scientific NanoDrop ND-2000 and was proceeded for cDNA synthesis.

3.12.4. cDNA Preparation

Prior to cDNA synthesis RNA was quantified on NanoDrop and concentration was determined. 10mM oligodT, RNA sample (according to concentration) and Nuclease free water were incubated at 65˚C for 5 minutes than placed on ice immediately and heat shock was given. After which 4 µl of *Reverse Transcriptase* (RT) buffer (Thermo Scientific), 0.5 RNAse Inhibitor (Thermo Scientific), 2 µl of 10 mM dNTPs and 1 µ of RT enzyme (Thermo Scientific) was added (Table 3.5) and incubated on 42˚C for 60 minutes and 70˚C for 10 minutes (Figure 3.4).

Table 3.5: Recipe for cDNA synthesis

Component	**Quantity(µl)**
10mM oligodT	1 µl
RNAse Inhibitor	0.5 µl
10mM dNTPs	2 µl
RT Buffer	4 µl
DEPC treated Water	Up to volume 20 µl
Reverse Transcriptase	1 µl
RNA	Depending on concentration
Total Volume	**20 µl**

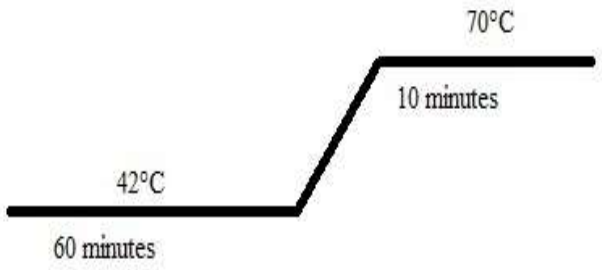


Figure 3.4: Program for cDNA synthesis

3.12.5. Primer Designing

Primer for qRT were obtained from literature (Table 3.6) and were tested for homology by UCSC genome browser InSilico PCR.

Table 3.6: Primer Sequences

Gene	Primer Sequence5' to 3'	Product size	Reference
ULK1 Forward Primer	CGTCCTCCAAGACGCTGTAT	100bp	(Lenhare *et al.*, 2017)
ULK1 Reverse Primer	CCTGTTGCTTTCCTCCAAAG		
Caspase3 Forward Primer	CGTGGTTCATCCAGTCCCTTT	102bp	(Liu *et al.*, 2017)
Caspase3 Reverse Primer	ATTCCGTTGCCACCTTCCT		
GAPDH Forward Primer	ACCCAGAAGACTGTGGATGG	175bp	(Ren *et al.*, 2016)
GAPDH Reverse Primer	CACATTGGGGGTAGGAACAC		
rbcL AF	ATGTCACCACAAACAGAGACTAA AGC	650bp	(Sun *et al.*, 2001)
rbcL AR	CTTTCTGCTACAAATAAGAATCG ATCTC		

3.12.7. Conventional PCR

For confirmation of synthesized cDNA a conventional PCR was done for GAPDH, the house-keeping gene. The conditions used are given in Figure 3.5. PCR constituents are listed in Table 3.7

Table 3.7: Recipe for conventional PCR

Constituent	Quantity (µl)
Nuclease-Free water	15
10mM Forward Primer	1
10mM Reverse Primer	1
2.5mM dNTPs	2
10X Taq Buffer	2.5
25mM $MgCl_2$	2
Taq Polymerase	0.5
cDNA	1
Total	**25**

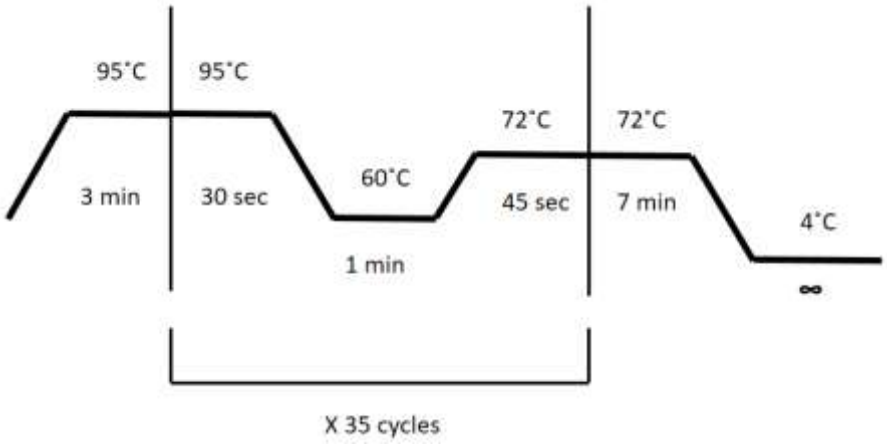


Figure 3.5: Conventional PCR Program

3.12.7. qRT-PCR

To obtain the CT values and expression analysis real time PCR was conducted using 25 µl of reaction mix containing 1 µl of each 10mM forward and reverse primer, 2.5 µl of cDNA and 12.5 µl of 2X SYBR Green/ROX qRT PCR reaction mix (Thermo Scientific). The program used for qRT-PCR for obtaining CT values of Caspase 3 and ULK-1 are shown in Figure 3.7 and 3.8, respectively. The program used for housekeeping gene, GAPDH, is shown in Figure 3.6.

Table 3.8: Recipe for Real-time PCR

Component	Quantity(µl)
2X SYBR Green/ROX qRT PCR reaction mix	12.5 µl
10mM forward primer	1 µl
10mM reverse primer	1 µl
Water	8.5 µl
cDNA	1 µg
Total Volume	**25 µl**

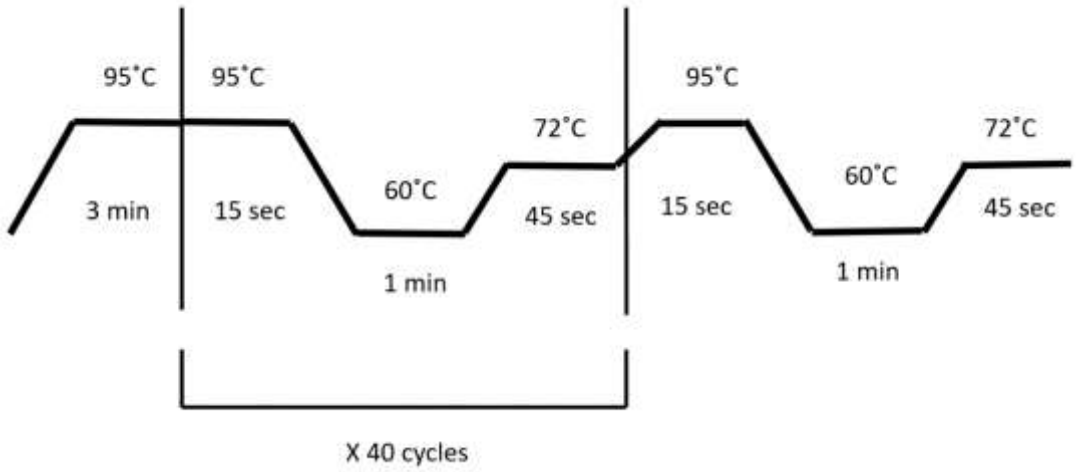


Figure 3.6: PCR Program for GAPDH amplification to obtain CT values at Tm= 60˚C with dissociation stage at the end.

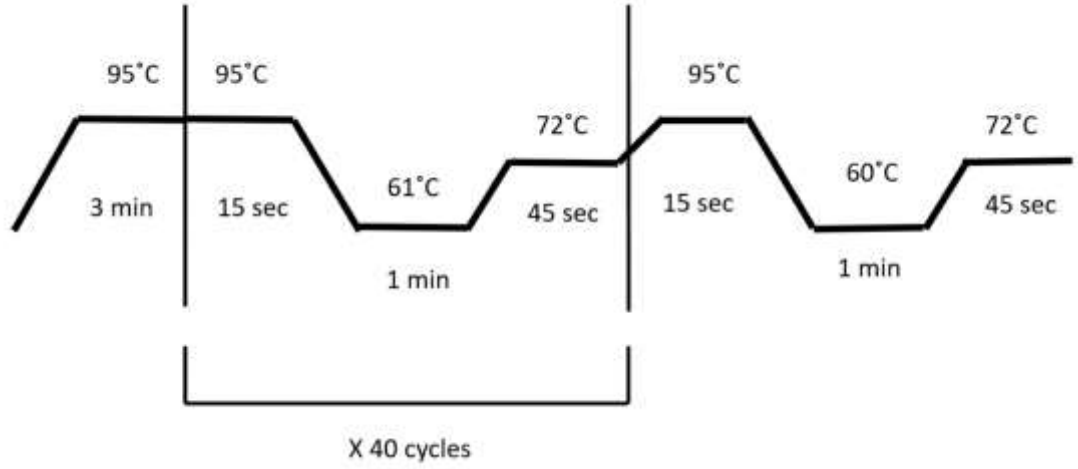


Figure 3.7: PCR Program for Caspase3 amplification to obtain CT values at Tm= 61˚C.

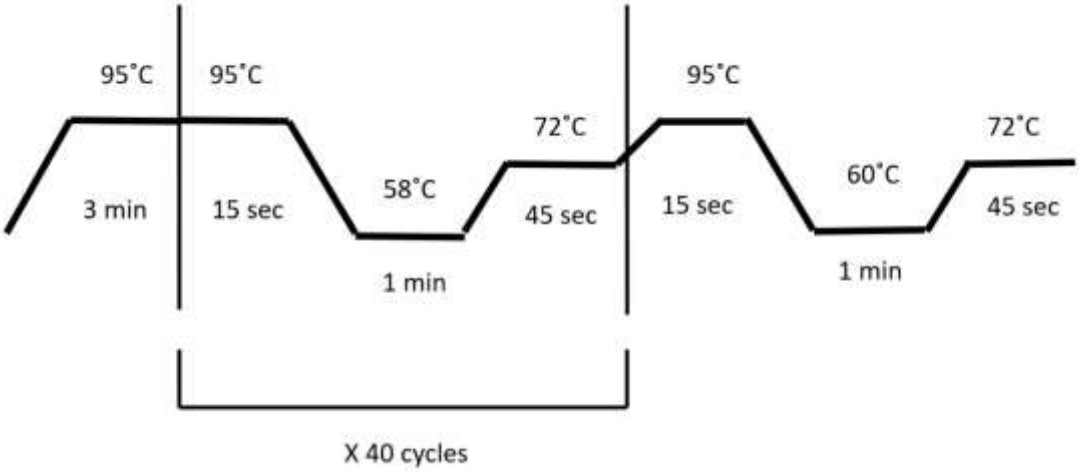


Figure 3.8: PCR Program for ULK-1 amplification to obtain CT values at Tm= 58˚C.

3.12.8. Expression Analysis

Expression analysis was done as describe previously by (Livak & Schmittgen, 2001). Expression analysis was performed by using ΔΔCt method also known as Livak method (Livak et.al, 2001).

ΔCt Calculation

ΔCt was calculated, in order to normalize the target gene, using the formula

$\Delta Ct = Ct_{\text{target Gene}} - Ct_{\text{Housekeeping Gene}}$.

Standard Deviation (SD)

SD for determination of the deviation from mean was calculated using the formula;

$$SD = \sqrt{SD\ Target Gene - SD\ Housekeeping\ Gene}$$

ΔΔCt Calculation

In order to determine ΔΔCt value the formula was determined by subtracting $\Delta Ct_{\text{treated}}$ from $\Delta Ct_{\text{untreated}}$; $\Delta\Delta Ct = \Delta Ct_{\text{treated}} - \Delta Ct_{\text{untreated}}$.

Fold change

To determine fold difference $2^{-\Delta\Delta Ct}$ was used.

3.13 Enzyme Linked Immuno-Sorbant Assay

ELISA was conducted by following the manual provided with kit by Elabscenences. Reagents and samples were taken out from refrigerator 20 minutes prior to commencing the experiment, to bring them at room temperature. 25X wash buffer was diluted to 1X working solution and different concentrations of reference standards were made. 100X biotinylated detection antibodies and 100X HRP conjugate were diluted to 1X working solution before bringing samples to room temperature. 100 µl of standard and sample were loaded in respective wells and then incubated for 90 minutes at 37˚C. All samples and reference standards were loaded in triplicate. Liquid was removed after incubation and 100 µl of bioyinylated antibody was added and left for 1 hour incubation at 37˚C. After which liquid was aspirated and washed 3 times with washing buffer and 100 µl of HRP conjugate was added followed by 30 minute incubation at 37˚C. 5 times washing was done with wash buffer after aspiration of liquid. 90 µl of substrate reagent was added and incubated for 15-30 minutes at 37˚C. 50 µl of stop solution was added to samples and OD value

was measured at 450 nm. Concentrations were determined by interpolating values against the standard curve.

3.14 Scanning Electron Microscopy

Skeletal muscles preserved in 10% formalin were gross-sectioned and dried until all the moister evaporated from the tissue and were loaded on spuds with the help of carbon tape. After which palladium-gold coating was done through sputter coater in which vacuum was built up to 10^{-1} Pa. Samples were then loaded onto sample loader and then placed in electron microscope machine and a voltage of 5KV was provided in order to avoid tissue damage and pictures were taken at various magnifications.

3.15 Statistical Analysis

Using GraphPad Prism and Microsoft Excel statistical analysis was performed. Student t test was performed to obtain individual p values in comparison with control population whereas two–way ANOVA was performed to check the association between variables. Graphs were made using GraphPad Prism version 7 for windows.

CHAPTER 4 CHAPTER 4: RESULTS

4.1 Plant Material Selection

Thymus serpyllum is a plant with ethnobotanical importance in Hunza and Nagar Valley (Figure 4.1) where people have been using it for years for the cure of multiple disorder. As it grows on high altitude it has plethora of phytochemicals that might be responsible to elicit an immune response to disorders.

Figure 4.1: Plant sampled from Rakaposhi base camp Hunza-Nagar Valley.

4.2 Plant Identification

4.2.1 Herbarium Sample Submission

Plant material was fixed on herbarium sheet and submitted to Pakistan Museum of National History under the voucher number 042852 (Figure 4.2).

Figure 4.2: Plant sample submitted for voucher number accusation.

4.2.2 Plant Barcoding

For molecular identification of plant DNA extraction was done shown in Figure 4.3. After which rbcL amplification was done in order to have molecular identification of the plant used during experiment (Figure 4.4). Maximum Likelihood was used to plot an unrooted tree that signified that plant had similarity with genus thymus (Figure 4.5). 0.09/base substitution was treated as scale distance. *Mentha longifolia* was taken as out group. The plant sequence obtained had sequence similarity with Thymus genus. There was sequence variation in the amplified product. The sequence was submitted on NCBI for obtaining gene accession number.

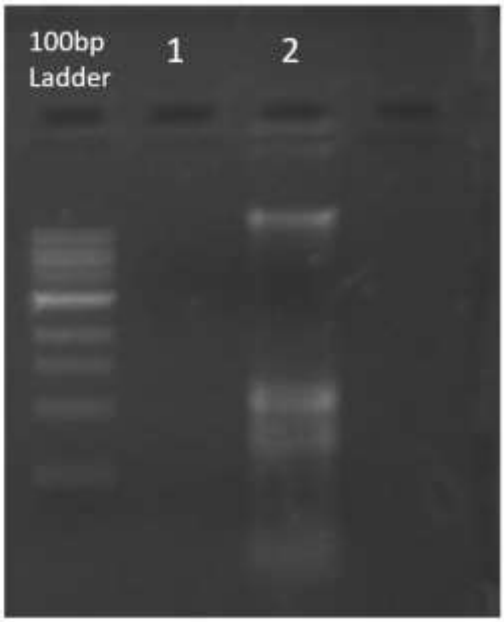


Figure 4.3: Representative gel image for Plant DNA extracted from *Thymus serpyllum*.

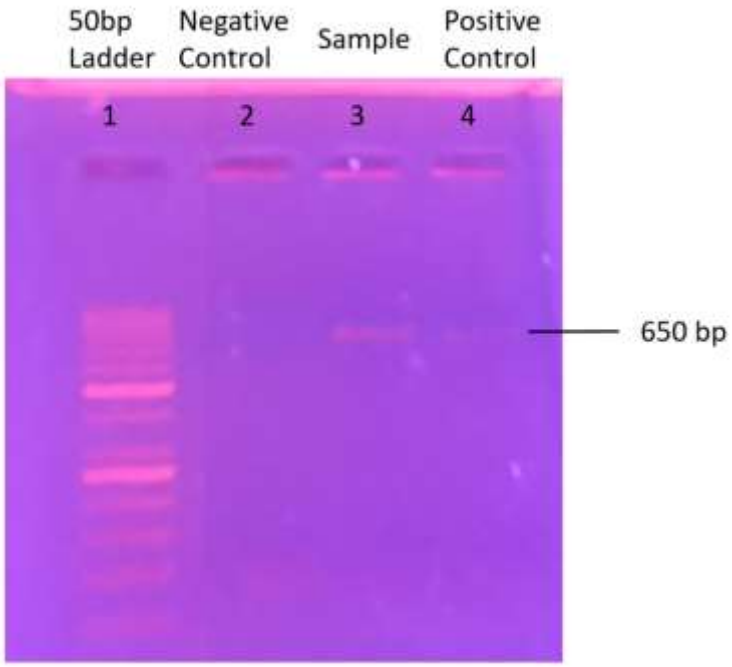


Figure 4.4: Amplified rbcL, the conserved region, for molecular identification.

The amplified product was 650bp. Lane 1 shows 50 bp ladder, Lane 2 shows negative control, Lane 3 is the amplified product from *Thymus serpyllum* whereas Lane 4 has positive control sample.

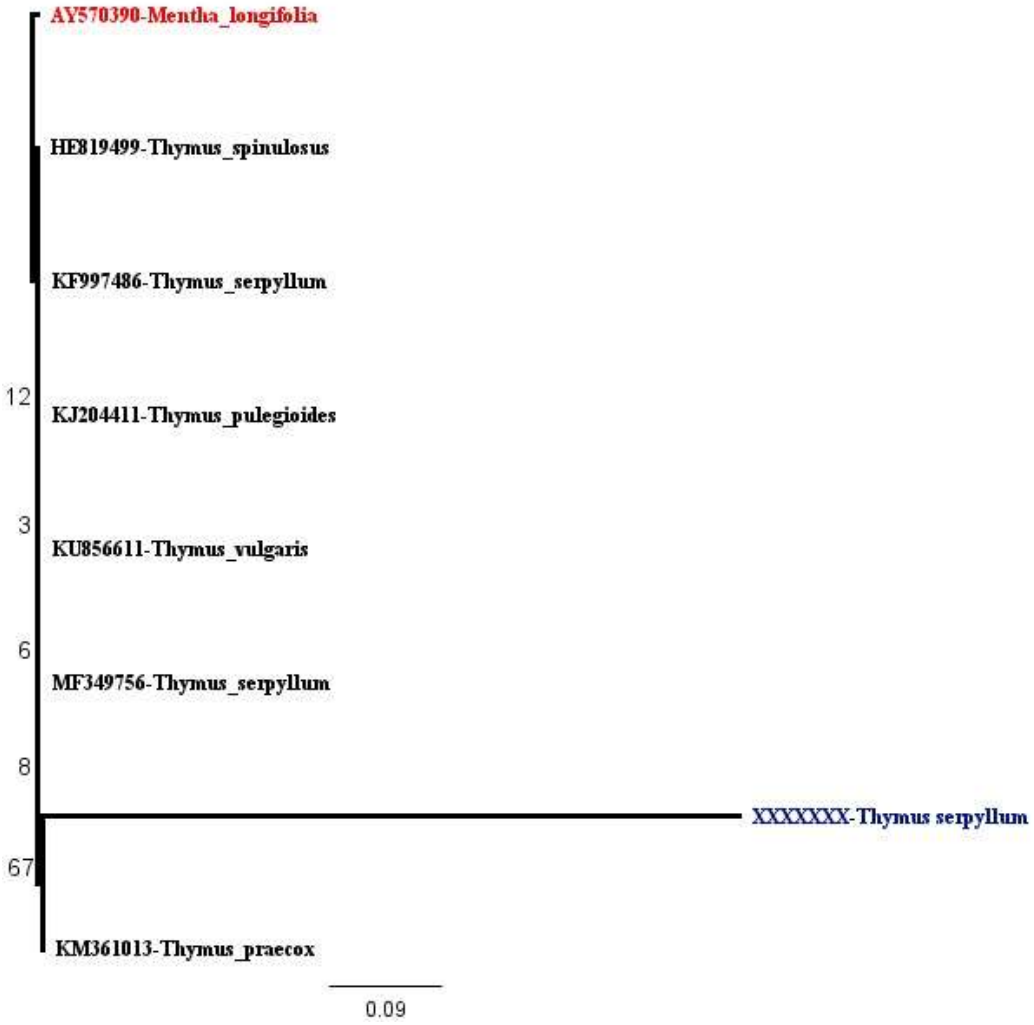


Figure 4.5: Phylogenetic tree was plotted with 0.09 scale that signified base substitution.

Mentha longifolia was used as an out group for identification. *Thymus serpyllum* showed homology with Thymus genus.

4.3 Plant Extract Characterization

4.3.1 Biochemical Test

In order to determine the constituents present in the extract 22 biochemical tests were conducted to determine phytochemical constituents present in ethyl acetate extract (Table 4.2) and aqueous extract (Table 4.1). Color change or physiological change was considered as a measure for the presence or absence of the compound. Figure 4.6 and Figure 4.7 show phytochemical analysis of aqueous extract whereas Figure 4.8 and Figure 4.9 show phytochemical analysis of ethyl acetate extract.

Table 4.1 Phytochemical constituents of Aqueous extract. Presence and absence of the compound was determined through color change and intensity of the color.

Sr.No	Test Name	Result	Interpretation
1	Alkaloids	-	No yellow precipitates were formed thus alkaloids were absent in the aqueous extract (Figure 4.6 (A)).
2	Phenols	+++	Brown precipitates formation shows presence of phenols in the aqueous extract (Figure 4.6 (B)).
3	Anthraquinones	++	Bluish Black coloration indicated the presence of anthoquinones in aqueous extract (Figure 4.6 (C)).
4	Flavonoids	+++	Formation of yellow precipitates indicate the presence of flavonoids in the aqueous extract (Figure 4.6 (D)).
5	Anthocyanins	-	No red coloration of the organic layer indicated absence of anthocyanins aqueous extract (Figure 4.6 (F)).
6	Leucoanthocyanins	-	No pinkish red coloration occurred and indicated absence of leucoanthocyanins in the aqueous extract (Figure 4.6 (E)).
7	Tannins	+++	Greenish to black coloration indicated the presence of tannins in aqueous extract (Figure 4.6 (G)).
8	Phlobatannins	+	Red precipitate formation depicted phalobatinnins in aqueous extract (Figure 4.6 (H)).
9	Coumarins	++	Yellow coloration indicated the presence of coumarins in aqueous extract (Figure 4.6 (I)).

10	Terpenoids	++	Deep red coloration was a mark for the presence of terpenoids in aqueous extract (Figure 4.6 (J)).
11	Diterpenes	+	Emerald green color appearance indicated the presence of diterpenes in aqueous extract (Figure 4.6 (K)).
12	Triterpenes	+	Lower layer turned yellow and indicated the presence of triterpenes (Figure 4.6 (L)).

Sr.No	**Test Name**	**Result**	**Interpretation**
13	Steroids	+++	Presence of reddish brown color at interface indicated the presence of steroids in the aqueous extract (Figure 4.7 (A)).
14	Sterols	+++	Appearance of red color in the lower layer showed presence of sterols in the aqueous extract (Figure 4.7 (B)).
15	Saponins	-	No froth formation indicated absence of saponins in aqueous extract (Figure 4.7 (C)).
16	Resins	++	Pinkish to red coloration indicated presence of resins in aqueous extract (Figure 4.7 (D)).
17	Glycosides	+++	Brown ring at the junction indicated the presence of glycosides in the aqueous extract (Figure 4.7 (E)).
18	Cardiac glycosides	+	Violet color formation below brown color indicated cardiac glycosides in aqueous extract (Figure 4.7 (F)).
19	Protein	+	No precipitation was formed and indicated absence of proteins in aqueous extract (Figure 4.7 (G)).
20	Amino acids	-	No violet color appeared and indicate absence of amino acids in aqueous extract (Figure 4.7 (H)).
21	Carbohydrates	++	No red precipitates were formed and showed absence of carbohydrates (Figure 4.7 (I)).
22	Deoxysugar	++	Formation of brown ring indicated the presence of deoxysugers (Figure 4.7 (J)).

Table 4.2 : Phytochemical constituents of Ethyl Acetate Extract. Presence and absence of the compound was determined through color change and intensity of the color

Sr.No	Test Name	Result	Interpretation
1	Alkaloids	++	Yellow precipitates were formed thus alkaloids were absent in the extract (Figure 4.8 (A)).
2	Phenols	++	Brown precipitates formation shows presence of phenols in the extract (Figure 4.8 (B)).
3	Anthraquinones	-	No Bluish Black coloration occurred indicating the absence of anthoquinones in extract (Figure 4.6 (C)).
4	Flavonoids	+++	Formation of yellow precipitates indicate the presence of flavonoids in the extract (Figure 4.8 (D)).
5	Anthocyanins	-	No red coloration of the organic layer indicated absence of anthocyanins extract (Figure 4.8 (F)).
6	Leucoanthocyanins	-	No pinkish red coloration occurred and indicated absence of leucoanthocyanins in the extract (Figure 4.8 (E)).
7	Tannins	++	Greenish to black coloration indicated the presence of tannins in extract (Figure 4.8 (G)).
8	Phlobatannins	-	Absence of Red precipitates depicted no phalobatinnins in extract (Figure 4.8 (H)).
9	Coumarins	+++	Yellow coloration indicated the presence of coumarins in extract (Figure 4.8 (I)).
10	Terpenoids	-	No deep red coloration was observed thus terpenoids were absent in extract (Figure

			4.8 (J)).
11	Diterpenes	+++	Emerald green color appearance indicated the presence of diterpenes in extract (Figure 4.8 (K)).
12	Triterpenes	+	Lower layer turned yellow and indicated the presence of triterpenes (Figure 4.8 (L)).

Sr.No	**Test Name**	**Result**	**Interpretation**
13	Steroids	-	Absence of reddish brown color at interface indicated the absence of steroids in the aqueous extract (Figure 4.9 (A)).
14	Sterols	++	Appearance of red color in the lower layer showed presence of sterols in the aqueous extract (Figure 4.9 (B)).
15	Saponins	+	Froth formation indicated presence of saponins in aqueous extract (Figure 4.9 (C)).
16	Resins	-	No Pinkish to red coloration indicated absence of resins in extract (Figure 4.9 (D)).
17	Glycosides	-	No Brown ring at the junction indicated the absence of glycosides in the extract (Figure 4.9 (E)).
18	Cardiac glycosides	++	Violet color formation below brown color indicated cardiac glycosides in extract (Figure 4.9 (F)).
19	Protein	-	No precipitation was formed and indicated absence of proteins in aqueous extract (Figure 4.9 (G)).
20	Amino acids	++	Violet color appeared and indicated presence of amino acids in extract (Figure 4.9 (H)).
21	Carbohydrates	-	No red precipitates were formed and showed absence of carbohydrates (Figure 4.9 (I)).
22	Deoxysugar	+	Formation of brown ring indicated the presence of deoxysugers (Figure 4.9 (J)).

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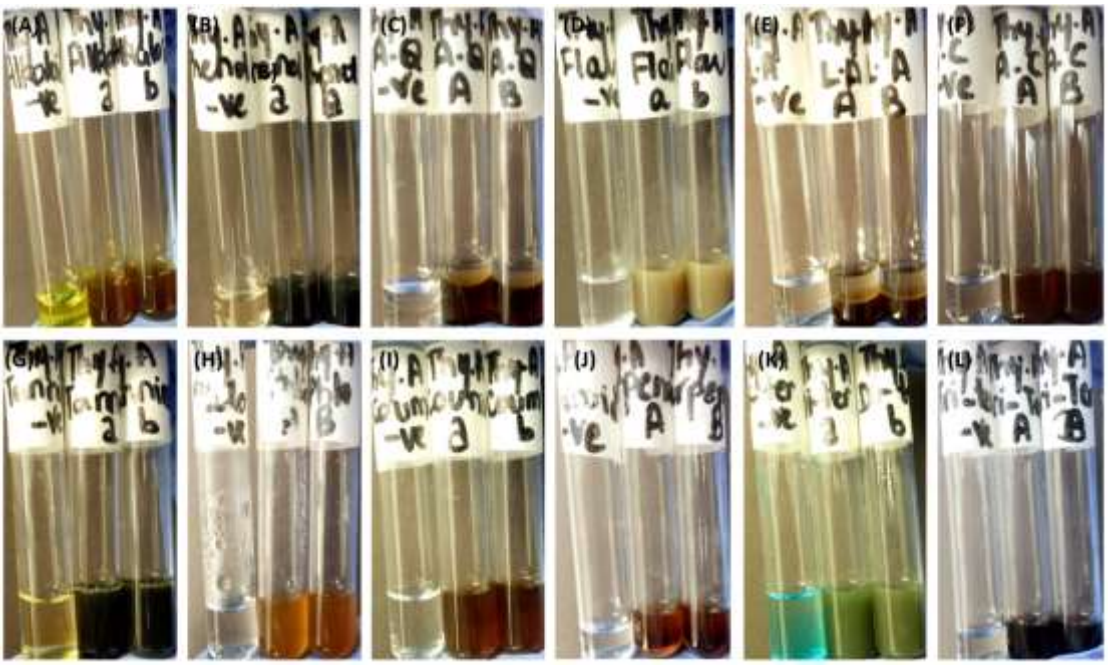

Figure 4.6: Phytochemical analysis of aqueous extract.

(A) No yellow precipitates were formed thus alkaloids were absent in the aqueous extract. **(B)** Brown precipitates formation shows presence of phenols in the aqueous extract. **(C)** Bluish Black coloration indicated the presence of anthoquinones in aqueous extract. **(D)** Formation of yellow precipitates indicate the presence of flavonoids in the aqueous extract. **(E)** No pinkish red coloration occurred and indicated absence of leucoanthocyanins in the aqueous extract. **(F)** No red coloration of the organic layer indicated absence of anthocyanins aqueous extract. **(G)** Greenish to black coloration indicated the presence of tannins in aqueous extract. **(H)** Red precipitate formation depicted phalobatinnins in aqueous extract. **(I)** Yellow coloration indicated the presence of coumarins in aqueous extract. **(J)** Deep red coloration was a mark for the presence of terpenoids in aqueous extract. **(K)** Emerald green color appearance indicated the presence of diterpenes in aqueous extract. **(L)** Lower layer turned yellow and indicated the presence of triterpenes.

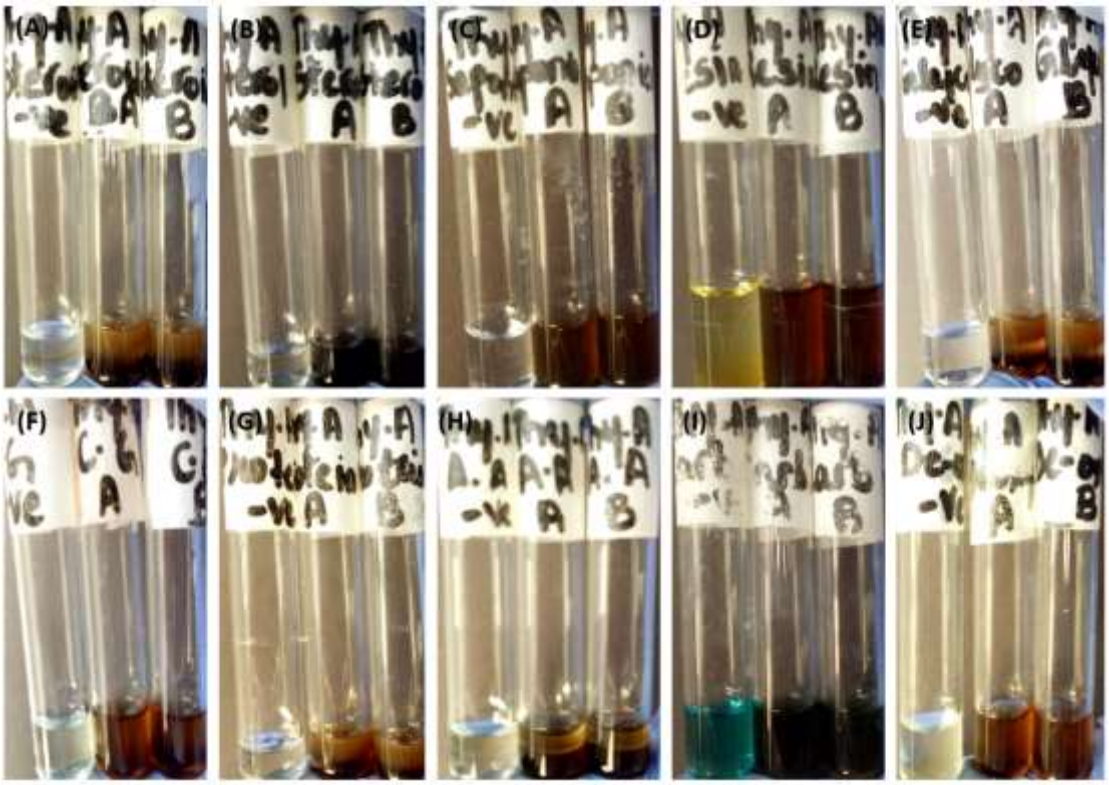

Figure 4.7: Phytochemical analysis of aqueous extract.

(A) Presence of reddish brown color at interface indicated the presence of steroids in the aqueous extract. **(B)** Appearance of red color in the lower layer showed presence of sterols in the aqueous extract. **(C)** No froth formation indicated absence of saponins in aqueous extract. **(D)** Pinkish to red coloration indicated presence of resins in aqueous extract. **(E)** Brown ring at the junction indicated the presence of glycosides in the aqueous extract. **(F)** Violet color formation below brown color indicated cardiac glycosides in aqueous extract. **(G)** No precipitation was formed and indicated absence of proteins in aqueous extract. **(H)** No violet color appeared and indicate absence of amino acids in aqueous extract. **(I)** No red precipitates were formed and showed absence of carbohydrates. **(J)** Formation of brown ring indicated the presence of deoxysugers.

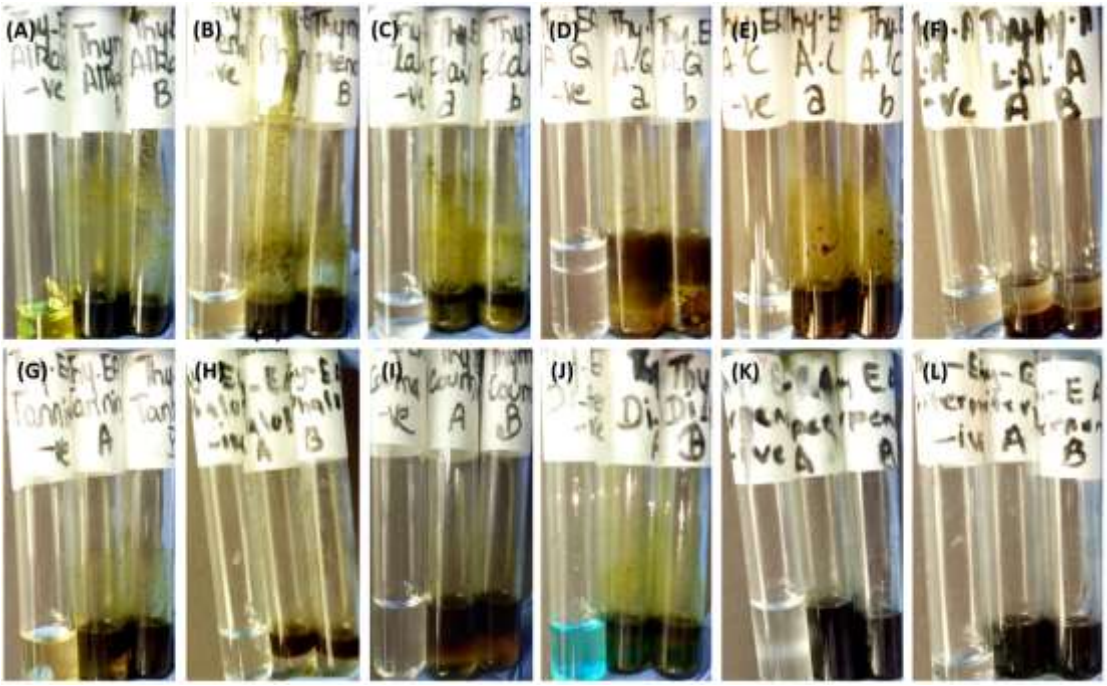

Figure 4.8: Phytochemical analysis of ethyl acetate extract.

(A) Yellow precipitates were formed thus alkaloids were present in the extract. **(B)** Brown precipitates formation shows presence of phenols in the extract. **(C)** No Bluish Black coloration occurred indicating the absence of anthoquinones in extract. **(D)** Formation of yellow precipitates indicate the presence of flavonoids in the extract. **(E)** No pinkish red coloration occurred and indicated absence of leucoanthocyanins in the aqueous extract. **(F)** No red coloration of the organic layer indicated absence of anthocyanins aqueous extract. **(G)** Greenish to black coloration indicated the presence of tannins in aqueous extract. **(H)** Absence of Red precipitates depicted no phalobatinnins in extract. **(I)** Yellow coloration indicated the presence of coumarins in aqueous extract. **(J)** No deep red coloration was observed thus terpenoids were absent in extract. **(K)** Emerald green color appearance indicated the presence of diterpenes in aqueous extract. **(L)** Lower layer turned yellow and indicated the presence of triterpenes.

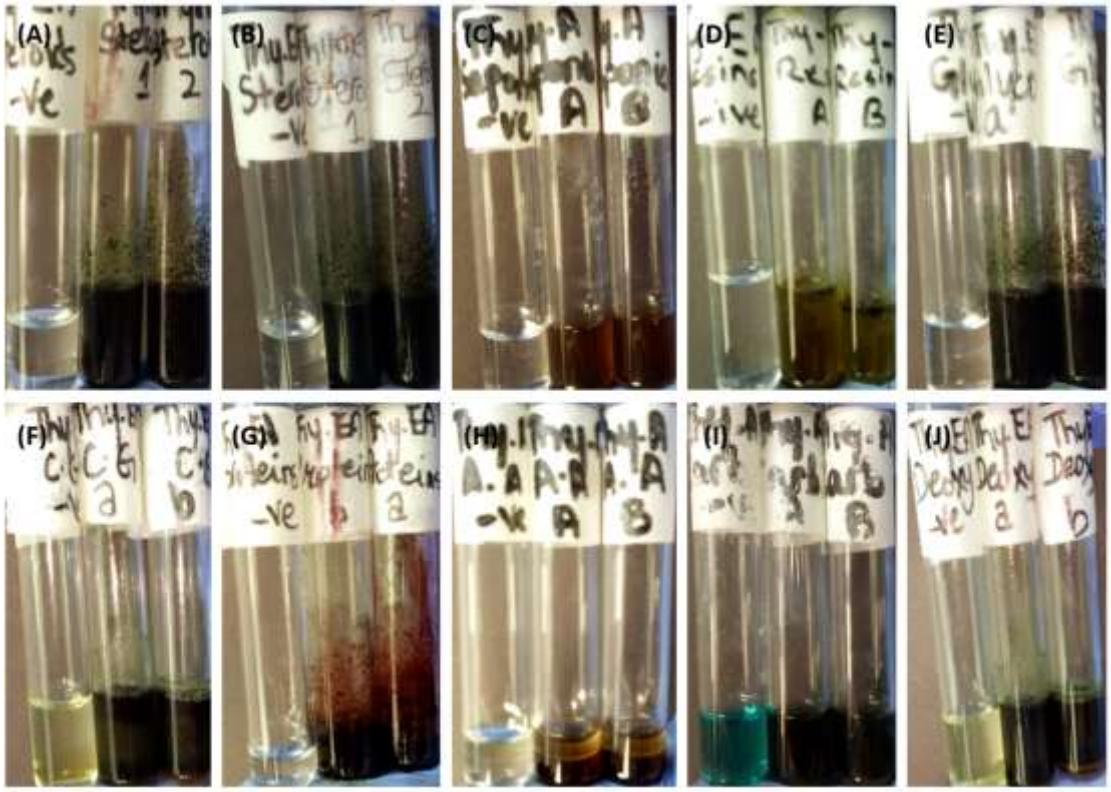

Figure 4.9: Phytochemical analysis of ethyl acetate extract.

(A) Absence of reddish brown color at interface indicated the absence of steroids in the extract. **(B)** Appearance of red color in the lower layer showed presence of sterols in the extract. **(C)** Froth formation indicated presence of saponins in extract. **(D)** No Pinkish to red coloration indicated absence of resins in extract. **(E)** No Brown ring at the junction indicated the absence of glycosides in the extract. **(F)** Violet color formation below brown color indicated cardiac glycosides in extract. **(G)** No precipitation was formed and indicated absence of proteins in aqueous extract. **(H)** Violet color appeared and indicated presence of amino acids in extract. **(I)** No red precipitates were formed and showed absence of carbohydrates. **(J)** Formation of brown ring indicated the presence of deoxysugers.

4.3.2 DPPH

In order to determine the antioxidant potential of the prepared plant extracts DPPH assay was conducted that exhibited an increasing pattern with the increase of concentration. A continuous trend was obtained in case of aqueous extract (Figure 4.10) plotted against the standard, ascorbic acid. At a concentration of 30µg/mL the ethyl acetate extract exceeded the percentage DPPH scavenging of the standard, ascorbic acid but then at 75µg/mL it was equivalent to that of standard (Figure 4.11). Figure 4.12 shows combined graph of ethyl acetate and aqueous extract free radical scavenging activity.

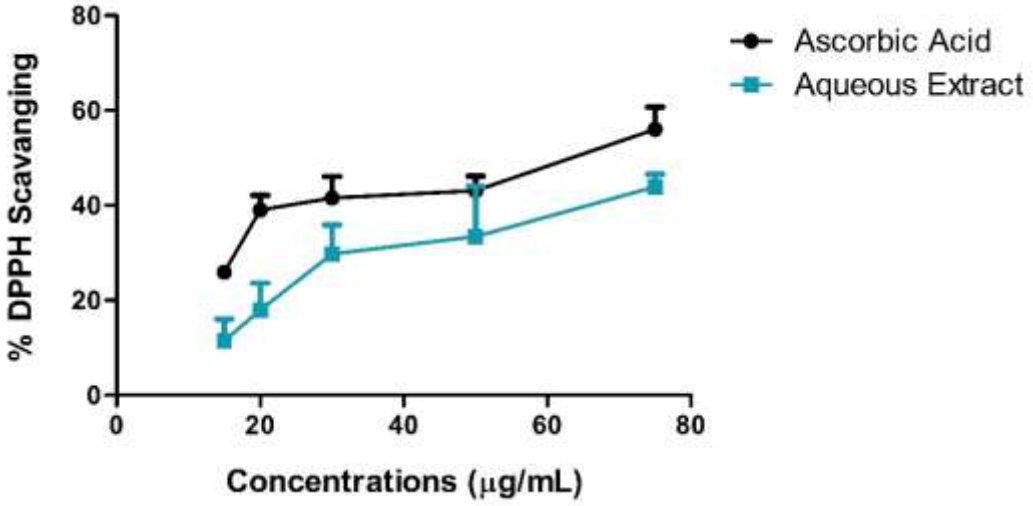

Figure 4.10: Percentage DPPH scavenging of aqueous extract.

The aqueous extract (turquoise line) exhibited an increase in free radical scavenging increased with the increasing concentration of the extract that was similar to that of the standard, ascorbic acid (black line).

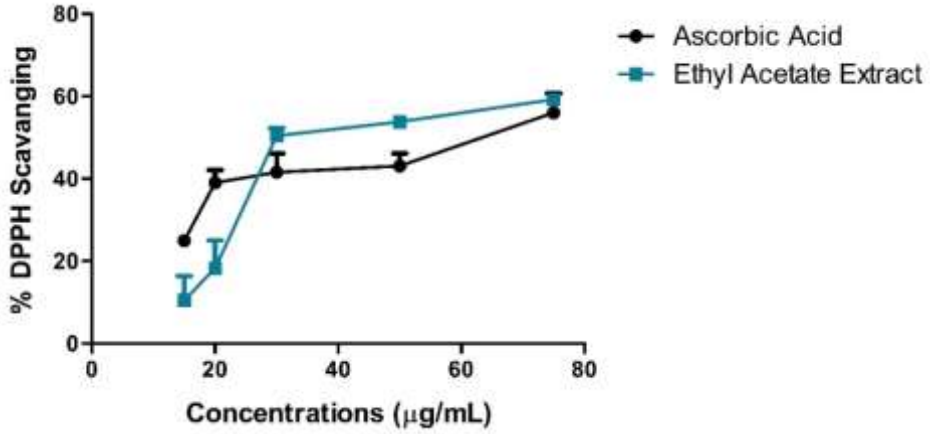

Figure 4.11: Percentage DPPH scavenging of ethyl acetate extract.

The ethyl acetate extract (turquoise line) exhibited an increase in free radical scavenging increased with the increasing concentration of the extract that exceeded that of the standard, ascorbic acid (black line). At higher concentrations it was almost equivalent to the standard.

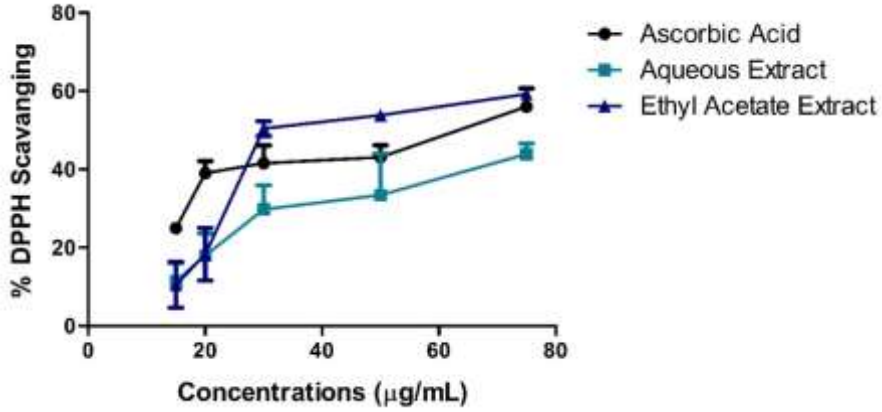

Figure 4.12: Comparison of % DPPH scavenging of ethyl acetate and aqueous extract.

The ethyl acetate extract (blue line) exhibited an increased free radical scavenging than aqueous extract (turquoise line). In comparison to aqueous extract, the ethyl acetate extract exhibited more free radical scavenging that even exceeded the standard, ascorbic acid (black line).

4.4 Measurement of Paw and Joint Edema

Paw size measurement was done from Day 0 to the Day of mice euthanization as a parameter for arthritis score mentioned in Table 3.5. The collagen induced arthritis (CIA) mimicked the RA pathogenesis thereby increasing paw depth and width (Figure 4.13).

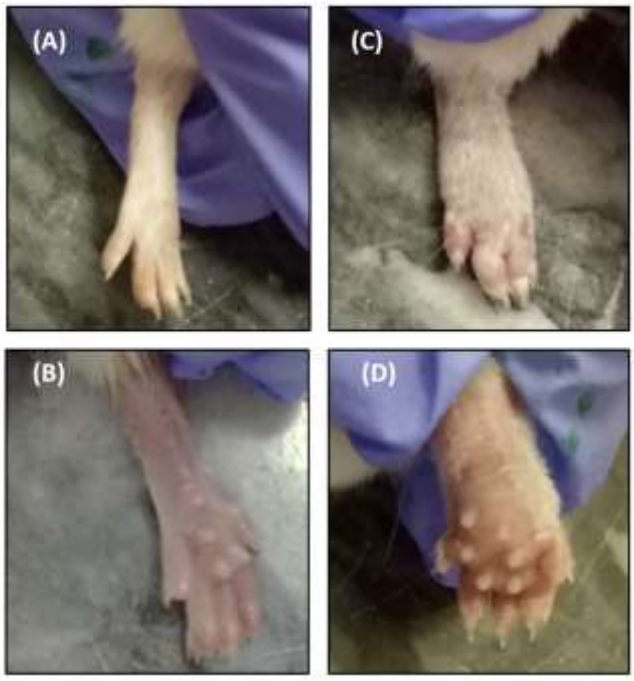

Figure 4.13: Representative image of arthritis induction taken on Day 21.

(A) Front view of normal paw of mice (arthritis score 0) **(B)** Back view of normal paw of mice (arthritis score 0) **(C)** Front view of Collagen type II Induced Arthritis (CIA) mice showing swelling and ankylosis in tarsals (arthritis score 4) **(D)** Back view of CIA mice showing swelling and abscess formation (arthritis score 4).

4.4.1 Arthritis Induction

During arthritis induction paw sizes were measured and it is evident from Figure 4.14 and Figure 4.15 that the paw depth and width increased significantly with the administration of immunization mixture. On Day 0, the primary day of immunization, paw sizes were equivalent but on Day 7 the paw depth increased ($p =< 0.0001$) from normal mice that did not receive the immunization mixture (Figure 4.11). From Day 7

to Day 28 the paw size increased to 0.6cm in paw width and 0.5cm in paw depth, thus confirming arthritis induction.

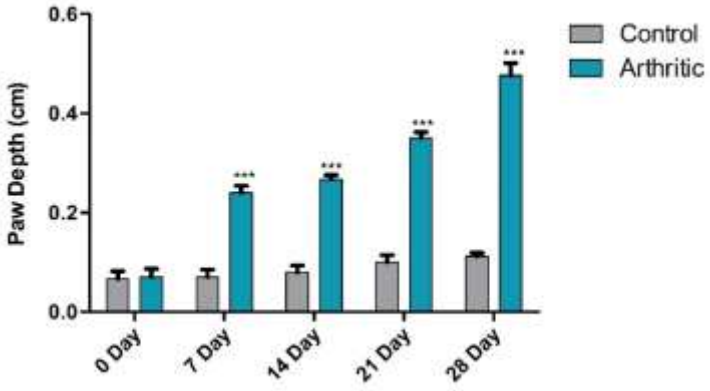

Figure 4.14: Paw depth during arthritis induction.

At Day 0 paws were at the same level (p=0.8735) whereas on Day 7 the paw depth significantly increased from normal (p=< 0.0001). Day 14, 21 and 28 paw depth increased from normal mice with progressive degeneration (p=< 0.0001, p=< 0.0001 and p=< 0.0001 respectively).

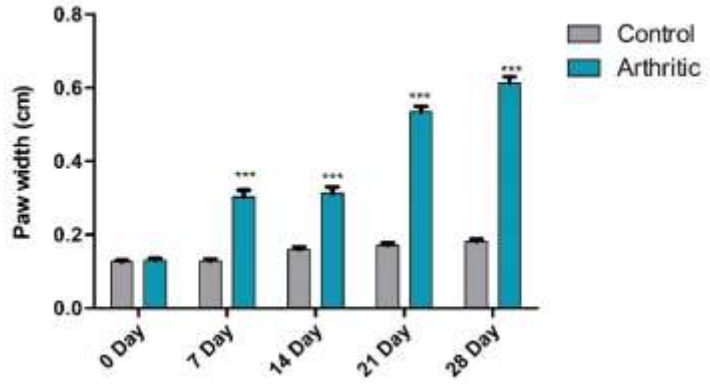

Figure 4.15: Paw width during arthritis induction.

At Day 0 paws were at the same level (p=0.8735) whereas on Day 7 the paw width significantly increased from normal (p=< 0.0001). Day 14, 21 and 28 paw width increased from normal mice with progressive degeneration (p=< 0.0001, p=< 0.0001 and p=< 0.0001 respectively).

4.4.2 Treatment

During week 1 of treatment paw sizes started to reduce but the paw size was greater than normal (p=< 0.0001) (Figure 4.16). Ethyl acetate treated mice model showed decrease in paw depth during week 3 (p=0.3456) whereas paw width was greater than normal (p=0.0223) (Figure 4.18). Aqueous extract produced its effect during week 4 paw depth reduced to almost normal (p=0.375) but paw width remained significantly greater than normal (p=0.0059) (Figure 4.19). During week 4 hydroxychloroquine treated mice showed decreased paw width (p=0.1535) but paw depth was significantly greater than normal (p=0.0058) (Figure 4.19). During week 5 the ethyl acetate extract treated and aqueous extract treated groups showed paw sized equivalent to normal (paw depth: p=0.146 and p=0.3402 respectively. Paw width: p=0.9803 and p=0.2718 respectively). The standard drugs produced their effect on week 5 the paw size started to reduce (Figure 4.20). During week 6 paw sizes of treated mice were equivalent to normal (Figure 4.21).

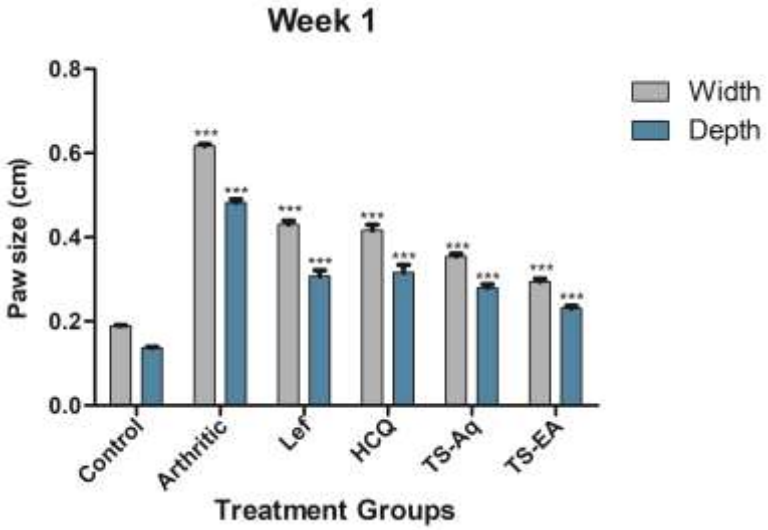

Figure 4.16: Paw size during treatment week 1.

Paw size of treatment groups reduced to normal but the arthritic group showed increased paw depth and paw width (p=< 0.0001). The paw depth and paw width (p= < 0.0001) of leflunamide treated group were significantly greater than normal. The paw depth (p= < 0.0001) and paw width (p= 0.0001) of hydroxychloroquine treated

mice was significantly higher than normal. The aqueous extract treated mice did not reduce paw width (p= < 0.0001) and paw depth (p= < 0.0001) to normal. The ethyl acetate extract treated mice the paw width (p= < 0.0001) and paw depth (p= < 0.0001) remained significantly higher than normal.

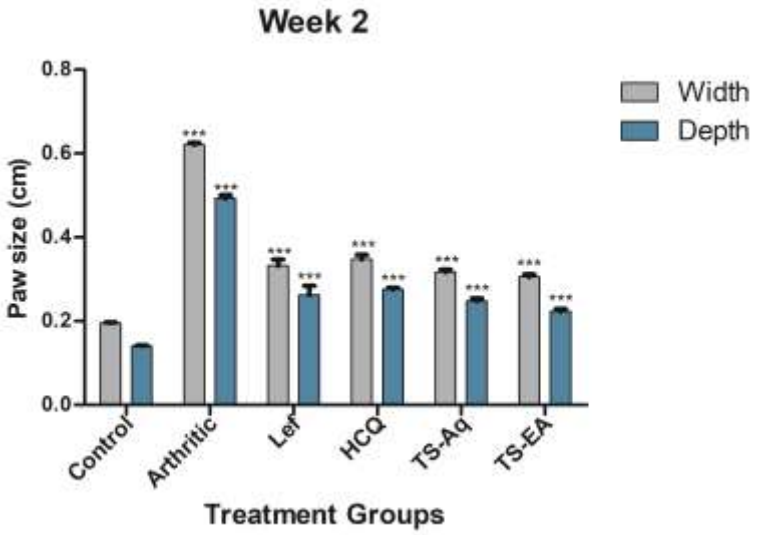

Figure 4.17: Paw size during treatment week 2.

Paw size of treatment groups reduced to normal but the arthritic group showed increased paw depth (p=< 0.0001) and paw width (p=< 0.0001). The paw depth (p= < 0.0001) and paw width (p= 0.0004) of leflunamide treated group were significantly greater than normal. The paw depth (p= < 0.0001) and paw width (p= 0.0001) of hydroxychloroquine treated mice was significantly higher than normal. The aqueous extract treated mice did not reduce paw width (p= < 0.0001) and paw depth (p= < 0.0001) to normal. The ethyl acetate extract treated mice the paw width (p= < 0.0001) and paw depth (p= < 0.0001) remained significantly higher than normal.

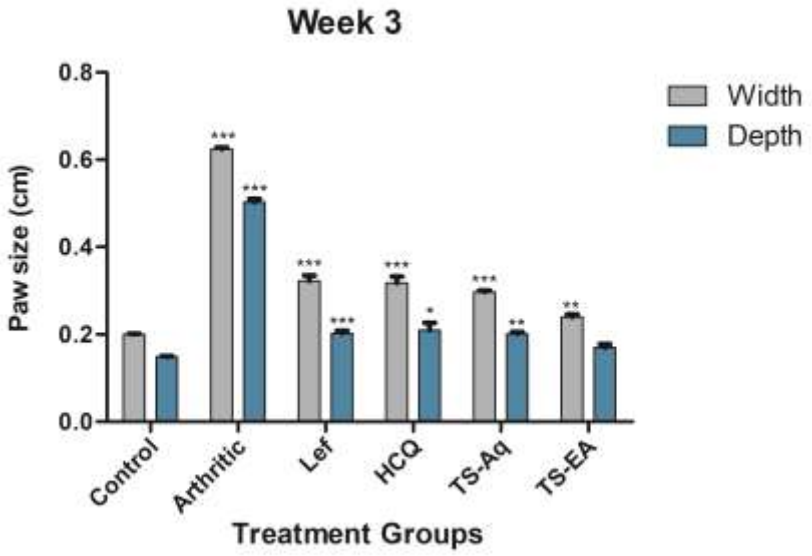

Figure 4.18: Paw size during treatment week 3.

Paw size of treatment groups reduced to normal but the arthritic group showed increased paw depth (p=< 0.0001) and paw width (p=< 0.0001). The paw depth (p= < 0.0001) and paw width (p= < 0.0001) of leflunamide treated group were significantly greater than normal. The paw depth (p= 0.0262) and paw width (p= 0.0001) of hydroxychloroquine treated mice was significantly higher than normal. The aqueous extract treated mice did not reduce paw width (p= < 0.0001) and paw depth (p= 0.0013) to normal. The ethyl acetate extract treated mice the paw width (p= 0.0223) remained significantly higher but the paw depth (p= 0.3456) reduced to normal.

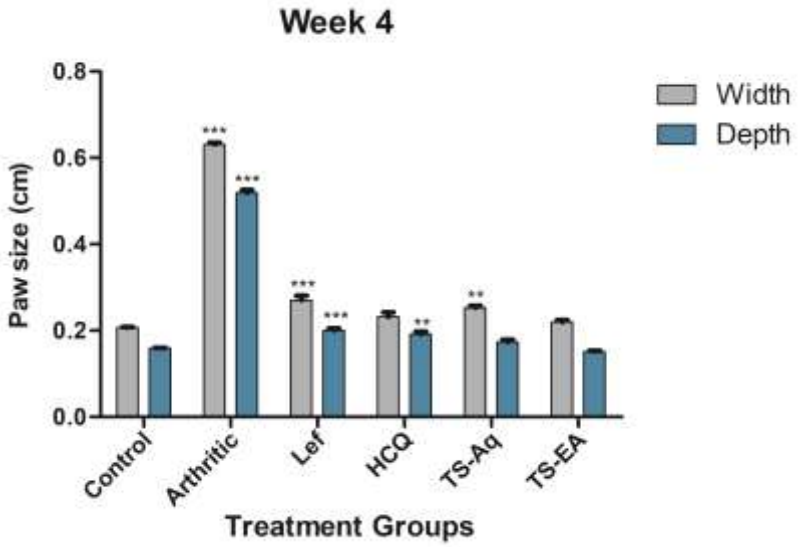

Figure 4.19: Paw size during treatment week 4.

Paw size of treatment groups reduced to normal but the arthritic group showed increased paw depth (p=< 0.0001) and paw width (p=< 0.0001). The paw depth (p= 0.0003) and paw width (p= 0.0007) of leflunamide treated group were greater than normal. But paw depth (p= 0.0058) was significantly higher than normal whereas paw width (p= 0.1535) of hydroxychloroquine treated mice was reduced to normal. The aqueous extract and ethyl acetate extract treated mice decreased paw width (p= 0.0059 and p= 0.3073 respectively) and paw depth (p= 0.375 and p= 0.1948 respectively) were equivalent to normal.

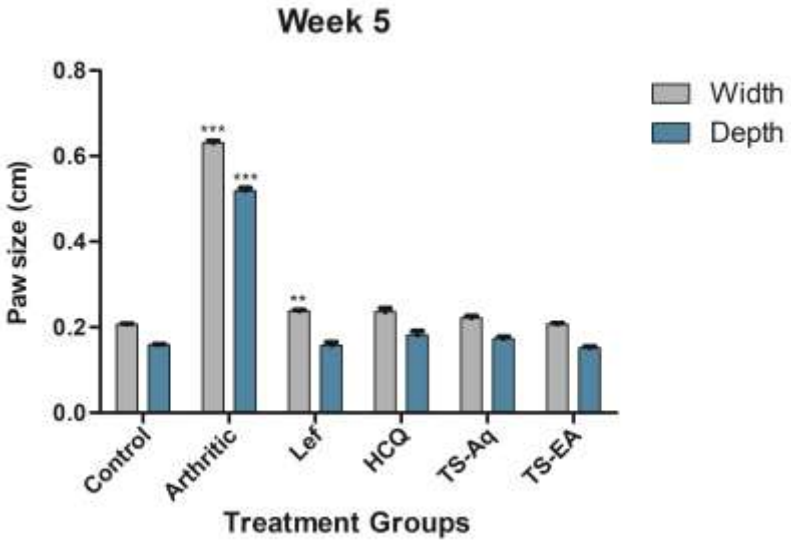

Figure 4.20: Paw size during treatment week 5.

Paw size of treatment groups reduced to normal but the arthritic group showed increased paw depth (p=< 0.0001) and paw width (p=< 0.0001). The paw depth (p=0.8746) and paw width (p=0.0025) of leflunamide treated group dropped close to normal and similar was the case with paw depth (p=0.112) and paw width (p= 0.0584) of hydroxychloroquine treated mice. The aqueous extract and ethyl acetate extract treated mice decreased paw width (p= 0.2718 and p= 0.9803 respectively) and paw depth (p= 0.3402 and p= 0.146 respectively) were equivalent to normal.

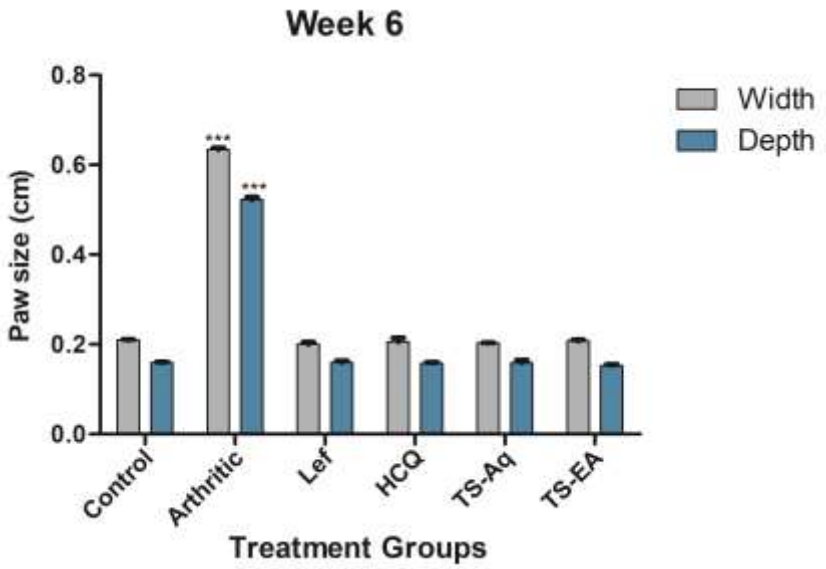

Figure 4.21: Paw width during 6 week treatment.

Paw size of treatment groups reduced to normal but the arthritic group showed increased paw depth (p=< 0.0001) and paw width (p=< 0.0001). The paw depth (p=1) and paw width (p=0.2296) of leflunamide treated group dropped near to normal and similar was the case with paw depth (p=0.7655) and paw width (p=0.7681) of hydroxychloroquine treated mice. The aqueous extract and ethyl acetate extract treated mice decreased paw width (p=0.0785 and p=0.0785 respectively) and paw depth (p=0.9555 and p=0.3734 respectively) were equivalent to normal.

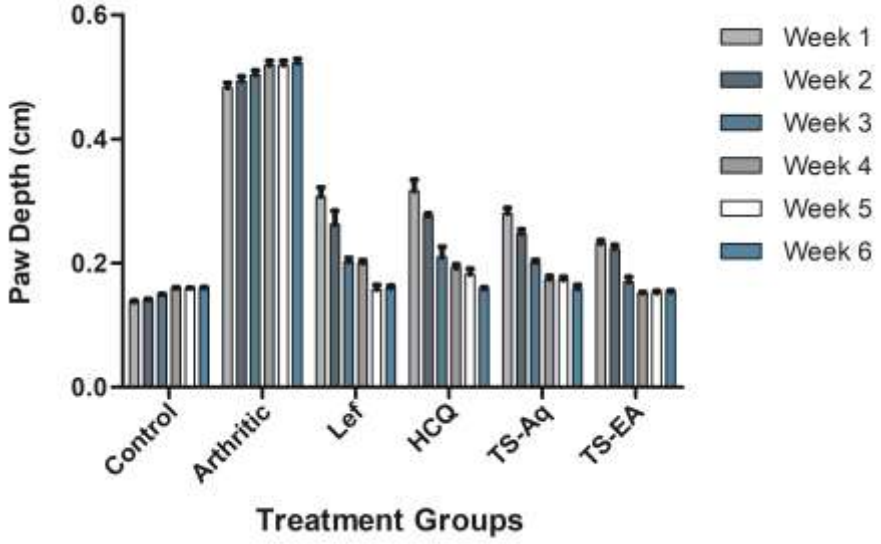

Figure 4.22: Summary of paw depth during 6 week treatment.

Paw depth of treated mice decreased than that of arthritic mice. The paw depth of treated mice was equivalent to normal mice that showed that treatment was effective.

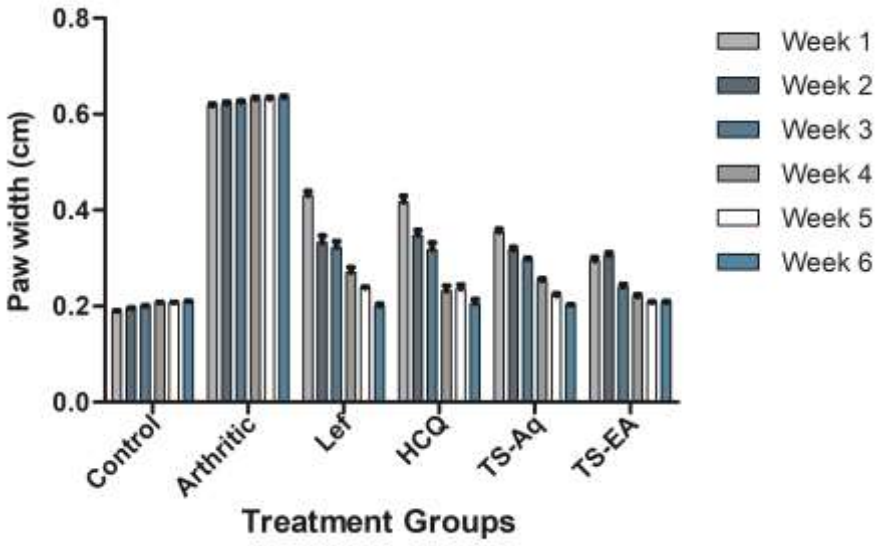

Figure 4.23: Summary of paw width during 6 week treatment.

Paw width of treated mice decreased than that of arthritic mice. The paw width of treated mice was equivalent to normal mice that showed that treatment was effective.

4.5 Spleen Indexing

Enlarged spleen is the characteristic feature of extra-articular manifestations of rheumatoid arthritis. Aqueous extract and ethyl acetate extract treated groups show less aggressive splenomegaly, which depicts that extracts directly act on reducing extra-articular manifestations. After arthritis induction the spleens increased in comparison to the normal mice. The mice to which treatment was administered did not show any deviation from the normal however the spleens of aqueous extract treated mice decreased and the ethyl acetate extract treated mice had lower splenomegaly or it went to reversal (Figure 4.24).

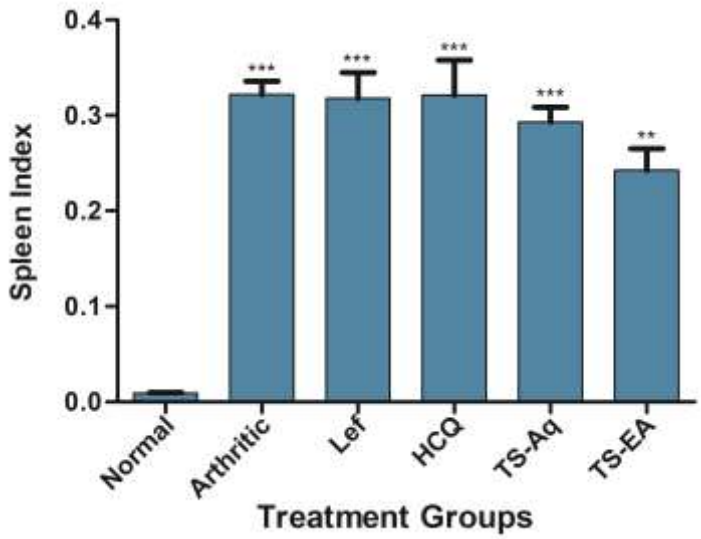

Figure 4.24: Spleen index after treatment.

Spleens of arthritic mice enlarged in comparison to normal mice (p=< 0.0001). Leflunamide and hydroxychloroquine treated mice retained the enlarged spleens (p=< 0.0001 and p=< 0.0001 respectively). The aqueous extract treated mice had less splenomegaly in comparison to arthritic mice but spleens were significantly greater than normal mice (p=< 0.0001) whereas ethyl acetate extract treated mice showed decreased splenomegaly (p=0.0012).

4.6 Histopathology

Histopathological analysis revealed morphological changes occurred after arthritis induction. Arrows indicate cell debris, joint degenration and modifcation whereas stars indicate cellular infiltraion. The arthritic mice had increased cellular infiltration that lead to bone erosion and joint remolding. Pannus formation is evident in Figure 4.25B. The extract treatment had protective effect on degenerating joint especially the aqueous extract whereas the standard drugs did not improve the joint degenration but the inflammation and hyperplasia were reduced.

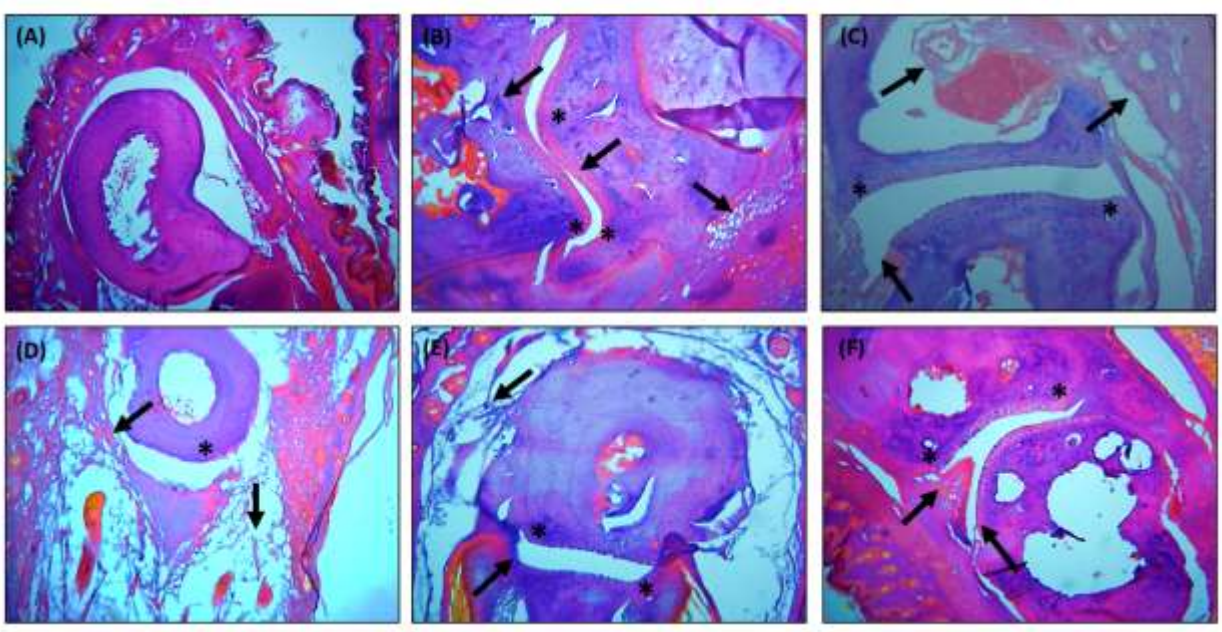

Figure 4.25: Histopathology for tarsal morphological analysis.

(A) Normal mice sample shows adequate joint spacing with intact boundaries expressing no neutrophil infiltration. **(B)** Joint architecture was distorted with bone erosion (arrows) and cellular infiltration (stars) **(C)** Hydroxychloroquine (standard drug) treated mice showed joint modification and continuous bone erosion. **(D)** Leflunamide (standard drug) treated mice showed joint modification and continuous bone erosion. **(E)** Aqueous extract treated mice indicated preservation of joint architecture and less cell infiltration **(F)** Ethyl Acetate extract treated mice indicated preservation of joint architecture but still there were some morphological changes and less cell infiltration.

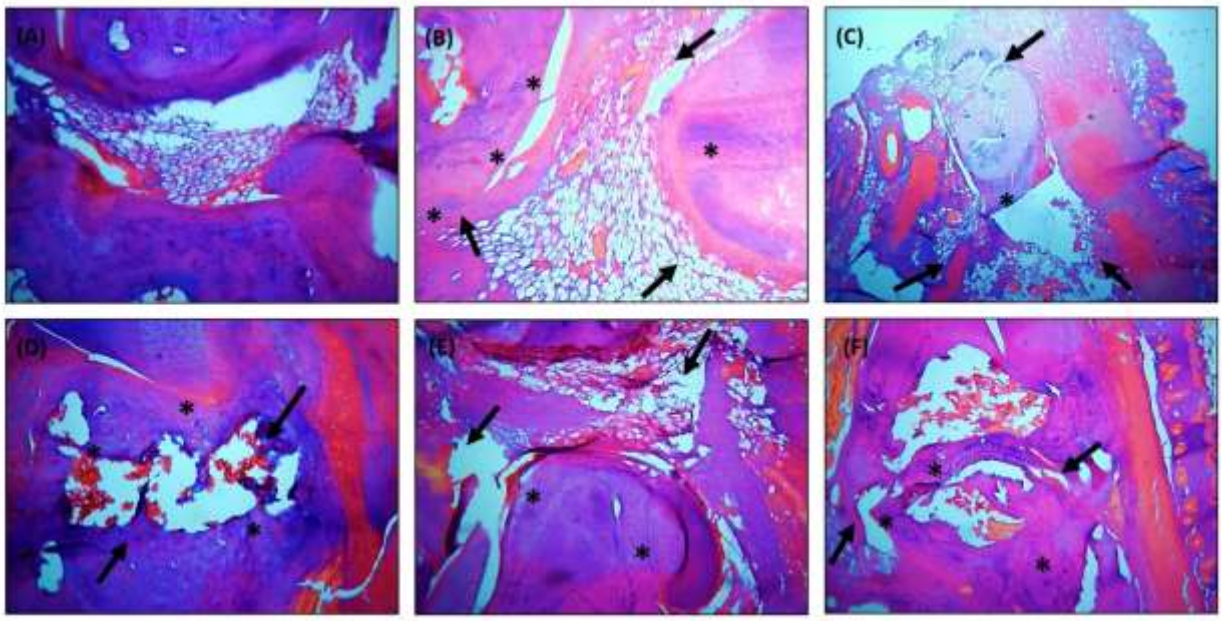

Figure 4.26: Histopathology for ankle joint morphological analysis.

(A) Normal mice sample shows adequate joint spacing with intact boundaries and cartilage expressing no neutrophil infiltration. **(B)** Joint architecture was distorted with bone erosion (arrows) and cellular infiltration (stars) **(C)** Hydroxychloroquine (standard drug) treated mice showed joint modification and continuous bone erosion. **(D)** Leflunamide (standard drug) treated mice showed joint modification and continuous bone erosion. **(E)** Aqueous extract treated mice indicated preservation of joint architecture and less cell infiltration **(F)** Ethyl Acetate extract treated mice indicated preservation of joint architecture but still there were some morphological changes and less cell infiltration.

4.7 ELISA

IL-6 levels were measured through ELISA and values were interpolated using standard curve (Figure 4.27). Arthritic group showed highest IL-6 serum levels significantly greater than normal ($p = < 0.0001$) whereas the treatment groups had reduced IL-6 levels. Leflunamide administration dropped IL-6 levels below normal ($p = 0.0605$) whereas the hydroxychloroquine and ethyl acetate treated extracts had slightly elevated IL-6 levels ($p = < 0.0001$ and $p = < 0.0001$ respectively). The aqueous

extract treated mice exhibited IL-6 levels equivalent to normal (p= 0.1171) (Figure 4.28).

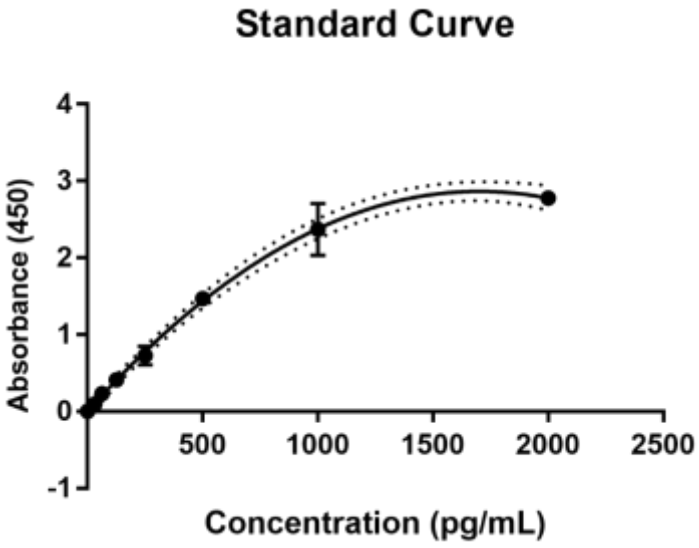

Figure 4.27: Standard curve used for interpolation of IL-6 concentrations.

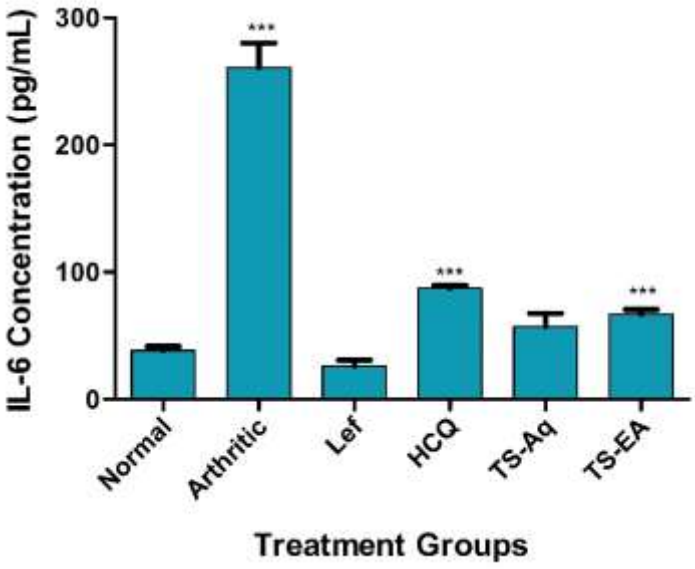

Figure 4.28: Comparison of IL-6 serum levels among treatment groups.

4.8 Expression Analysis

4.8.1 Conventional PCR

Confirmation of cDNA synthesis was done through GAPDH amplification via RT-PCR (Figure 4.29).

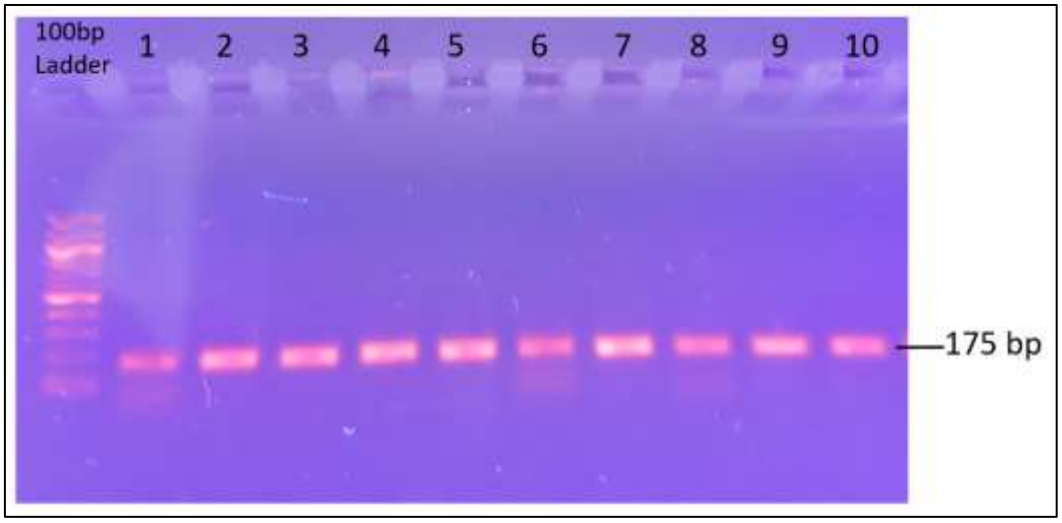

Figure 4.29: Confirmation of cDNA synthesis by GAPDH amplification.

Lane has 100bp Ladder. Lane 1 has positive control whereas Lane 2-10 show GAPDH amplification of 175bp.

4.8.2 Standard Curve Analysis

Dilution for real time PCR was optimized through standard curve analysis. Mean Ct value with deviation of triplicate was plotted on y-axis while cDNA concentrations were plotted on x-axis. cDNA dilution in NF water with 1/5 strength was chosen as amplification plot, dissociation and standard deviation in triplicate was most favorable. Moreover, for relative gene expression only those ct value were selected which showed standard amplification as shown in Figure 4.30 and standardized dissociation curve (for example in Figure 4.31) for respective gene.

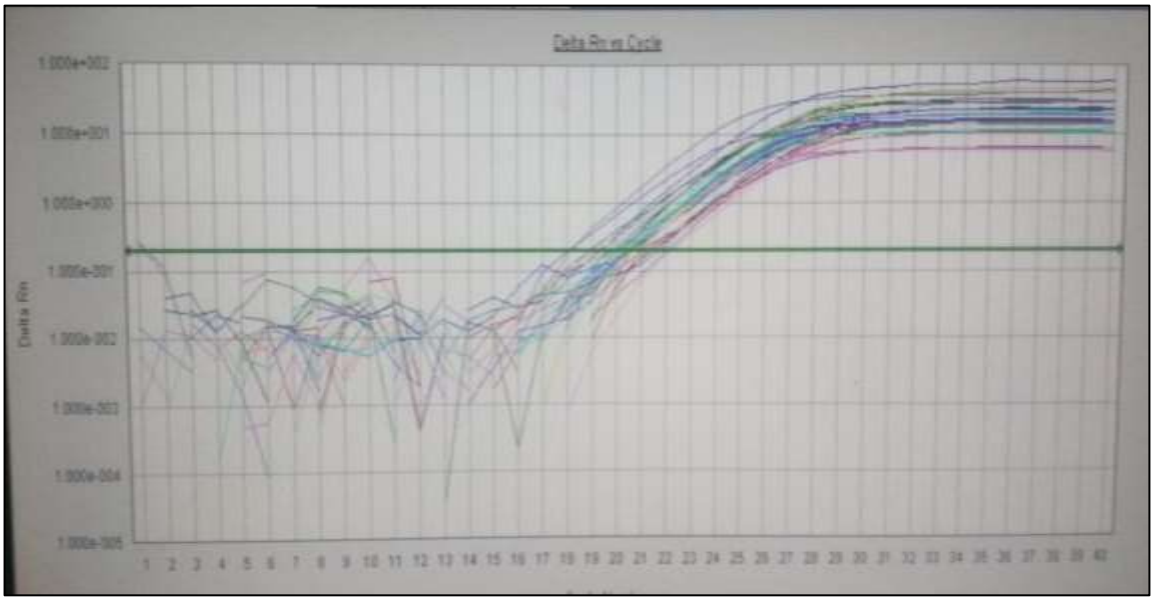

Figure 4.30: Amplification plot for GAPDH gene

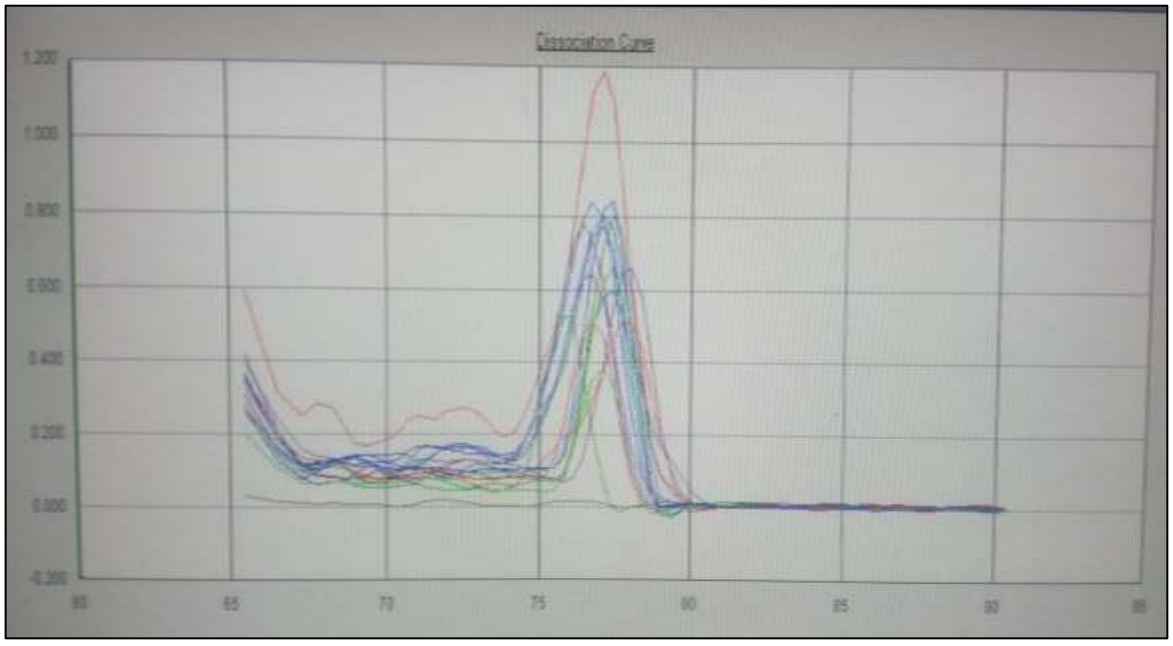

Figure 4.31: Dissociation Curve for GAPDH gene

4.8.3 Expression Analysis

Expression analysis revealed the down regulation of ULK-1, the autophagy marker in arthritic and leflunamide treated group. The hydroxychloroquine and aqueous extract treated mice had their ULK-1 upregulated to normal whereas ethyl acetate extract treated mice had significantly upregulated ULK-1 fold increase. Caspase 3 expression was upregulated to almost 5 fold in leflunamide treated group and approximately 1.7 fold upregulation in ethyl acetate extract treated group. Hydroxychloroquine and aqueous extract treated mice depicted approximately 2.14 and 1.74 fold increase in casepase 3 expression.

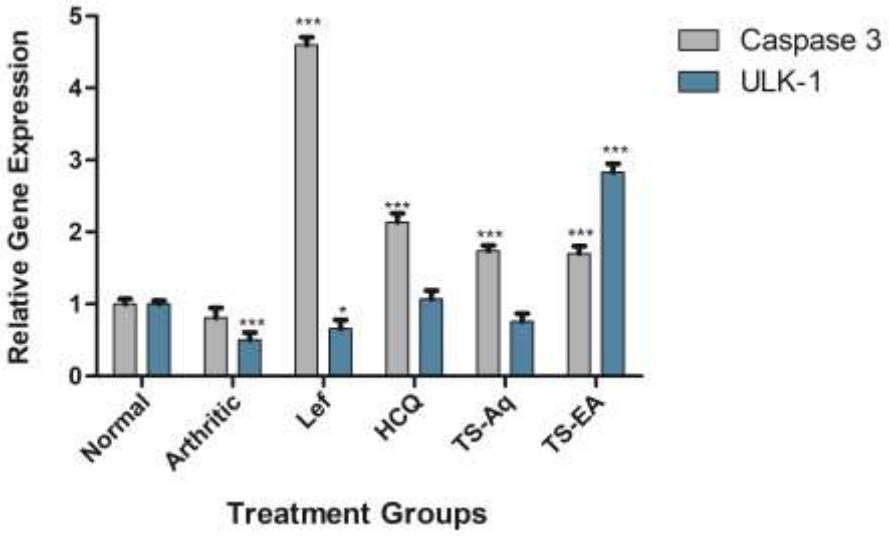

Figure 4.32: Relative gene expression of autophagy and apoptotic genes.

4.9 Immunohistochemistry

Immunohistochemitry was done to evaluate LC3 expression that was upregulated in arthritic sample whereas the treatment groups showed variable expression of LC3. Leflunamide treated group showed low LC3 levels whereas hydroxychloroquine inhibited LC3 to normal. The extract treated groups has low LC3 expression in skeletal muscles Figure 4.33.

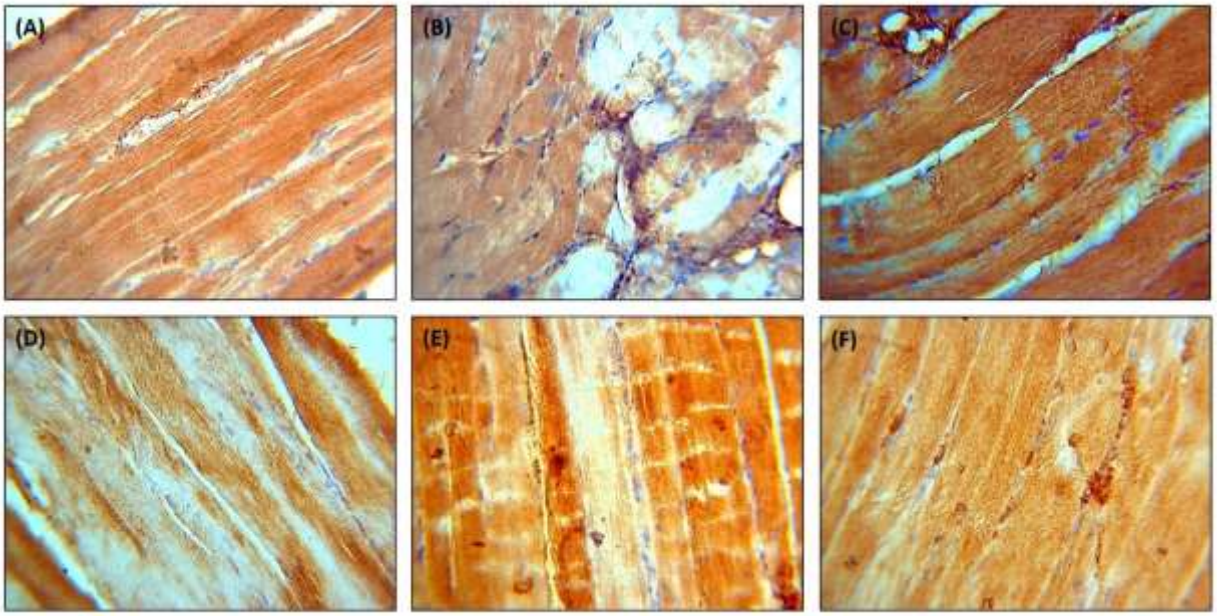

Figure 4.33:Representative Image of immunohistochemistry exhibiting LC3b expression in skeletal muscle cells.

(A) Normal mice sample showing normal expression of autophagy marker **(B)** Increased LC3b expression in arthritic sample **(C)** Hydroxychloroquine (standard drug) acted as inhibitor or LC3b and reduced its expression **(D)** Leflunamide (standard drug) also reduced the level of protein thereby interfering with autophagic pathway **(E)** Aqueous extract of *Thymus serpyllum* significantly reduced the LC3b expression restoring it to normal level **(F)** Ethyl Acetate extract reduced the LC3b expression as well but it molded the expression in order to restore homeostasis.

4.10 Electron Microscopy

Cell structure and morphology was evaluated through scanning electron microscopy. Normal myofibrils without bleb formation was observed in control group whereas arthritic group depicted muscular degeneration and confirmation of increased cellular autophagy. The leflunamide treated group, aqueous extract treated group and hydroxychloroquine treated group depicted bleb formation. Ethyl acetate extract treated mice showed signs of autophagy along with apoptosis (Figure 4.24 and Figure 4.25).

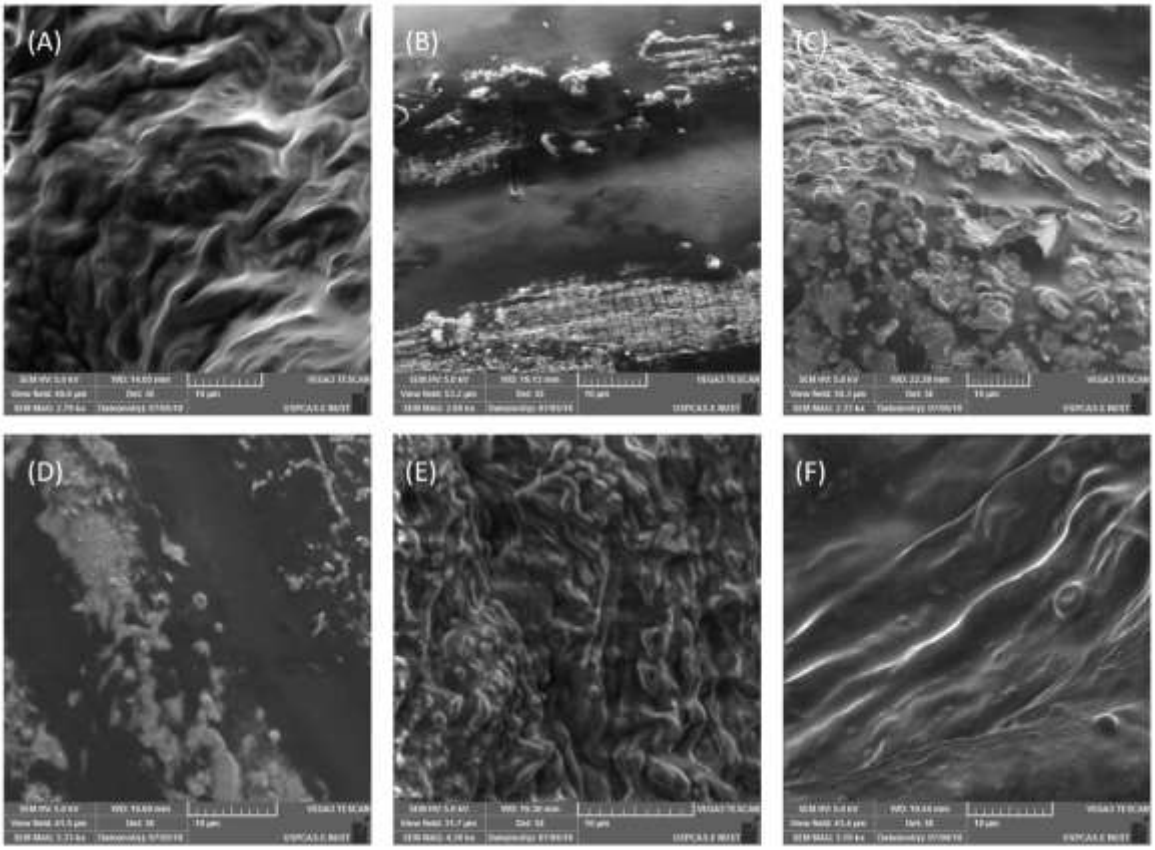

Figure 4.34: Representative image of scanning electron microscopy of skeletal muscle cells exhibiting muscle morphology and structure.

(A) Normal mice sample showed normal myofibrils with no tissue degeneration **(B)** Muscle degeneration and disruption can be evidently inferred from the micrograph **(C)** Hydroxychloroquine (standard drug) showing blebing on tissue surface **(D)** Leflunamide (standard drug) showing muscle distruption and evident signs of apoptosis **(E)** Aqueous extract of *Thymus serpyllum* also exhibits normal morphology and signs of apoptosis **(F)** Ethyl Acetate extracts shows signs of autophagy.

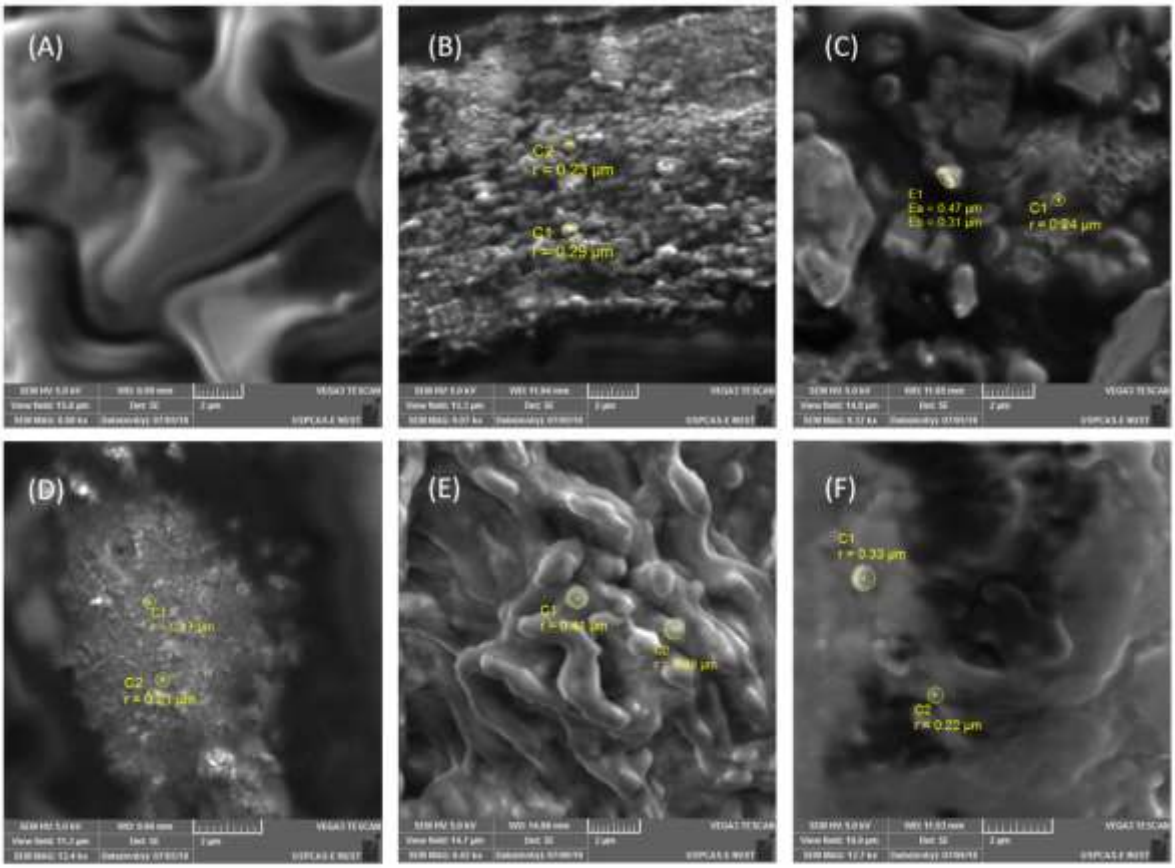

Figure 4.35: Representative image of scanning electron microscopy of skeletal muscle cells exhibiting muscle morphology and structure.

(A) Normal mice sample showed no bleb formation **(B)** Muscle of arthritic mice model showing engulfing of the debris **(C)** Hydroxychloroquine (standard drug) induced apoptosis by reducing autophagy **(D)** Leflunamide (standard drug) exhibited bleb formation and debris clearance **(E)** Aqueous extract of *Thymus serpyllum* showed bleb formation indicating apoptosis **(F)** Ethyl Acetate extracts exhibited increased apoptosis along with autophagy induction.

CHAPTER 5 : DISCUSSION

RA is an autoimmune disorder with mysterious pathophysiology. This multifactorial disorder is characterized by bone erosion, deformation and dislocation that lead to risk of immobility and fractures which is attributed to the malfunctioning of osteoclasts, under the influence of pro-inflammatory cytokines and chemokines, that hinder with the activity of osteoblasts (Gough *et al.*, 1994; Gravallese *et al.*, 2015; McInnes & Schett, 2011; Schett & Gravallese, 2012; Scott *et al.*, 2000). Auto-antibodies, ACPAs and RF, are produced in response to the citrullinated proteins and evoke immune response (Vossenaar *et al.*, 2003). ACPAs and self-proteins such as citrulinated collagen type II, histone, fibrinogen and etc form a complex that leads to complement system activation and lead to production of pro-inflammatory cytokines (Anquetil *et al.*, 2015; Sabharwal *et al.*, 1982; Zhao *et al.*, 2008). As a result of altered glycosylation, neo-antigen and Fc-receptor engagement lead to bone loss via TLR mediated macrophage activation or osteoclast activation (Harre *et al.*, 2012; Kerkman *et al.*, 2015; Rombouts *et al.*, 2013; Rombouts *et al.*, 2015). On the other hand, RFs have direct involvement in cytokine production and macrophage activation (Anquetil *et al.*, 2015; Sokolove *et al.*, 2014). The lymphocyte and cytokine infiltration instigates synovial hyperplasia, progressive bone deformation and other arthritic symptoms (Lundy *et al.*, 2007). These macrophages and dendritic cells are involved in antigen presentation, maintenance of immunity, T-cell homeostasis and synovial inflammation (Farache *et al.*, 2013; Galli *et al.*, 2011; Geissmann *et al.*, 2010) as circulating activated monocytes, expressing Toll-Like Receptors (TLRs), move into the synovial cavity where the differentiate into macrophages which produce enzymes, cytokines and chemokines that contribute to the destruction of cartilage, bone and synovial inflammation (Choy & Panayi, 2001; Q. Huang *et al.*, 2007; Q. Huang & Pope, 2010; van den Berg & van Lent, 1996).

Treatment options for rheumatoid arthritis do not cure but alleviate the symptoms and slows the progression of the disorder. NSAIDs are used to cure acute inflammation whereas steroids, DMARDs such as leflunamide and Biologics are used with increasing disease severity. This increasing disease severity imparts treatment

resistance that leads to poor disease management. In order to have better treatment options for disease cure the study was conducted to evaluate the ethno-botanical herbs being used for the treatment of chronic inflammatory conditions such as RA. Tomorou, *Thymus serpyllum,* is an indigenous plant from Hunza-Nagar Valley that is ethnobotanically used for the treatment of respiratory and gastrointestinal problems (Jabeen *et al.,* 2015). Thus due to its use against inflammatory problems the study aimed to evaluate the anti-inflammatory effect of 'tomoru' and to unravel the underlying mechanism that complicates the pathogenesis of RA.

In order to check the therapeutic efficacy of the plant, extracts were prepared using ethyl acetate and water as solvents for isolation of non-polar and polar compounds respectively. The phytochemical analysis revealed the presence of flavonoids, steroids, terpenoids and polyphenols as components of both extracts that was in accordance with the literature as all of these components are part of thyme essential oils (Jarić *et al.,* 2015; Kulišić *et al.,* 2007). The prepared extracts were administered to CIA mice model in feed along with two standard drug groups. Mode of administration of standard drugs and plant extracts prepared was oral as it had least toxicity. Hydroxychloroquine and leflunamide were given as standard drugs due to their properties of targeting autophagy and apoptotic markers. Paw size measurement revealed the effect of treatment being given to CIA mice model and variation of paw width and depth from normal and arthritic mice.

Extra-articular manifestations are a characteristic feature of progressing RA. As a result of disease prognosis vasculitis, interstitial lung disease, cardiovascular disorders, lymphoma, and secondary amyloidosis are associated with RA and the characteristic feature is spleen enlargement (Choi *et al.,* 2002; Hurd, 1979; Jacobsson *et al.,* 2007; H. H. Lee *et al.,* 2007; Listing *et al.,* 2013; Patatanian & Thompson, 2002). Spleen indexing revealed spleen enlargement and other comorbid conditions in arthritic mice that were not provided treatment. Although the standard drugs revealed alleviation of arthritic symptoms thereby implying that disease progression was decelerated but splenomegaly remained constant. No observable difference was observed in spleens of arthritic, leflunamide treated and hydroxychloroquine treated mice models. However extract administration revealed less aggressive splenomegaly

especially in ethyl acetate extract treatment significantly alleviated the arthritic symptoms along with reducing spleen in size this was may be due to high anti-oxidant activity of the extract. Whereas the aqueous extract demonstrated a less prompt effect in comparison to the ethyl acetate extract. Splenomegaly was assuaged in case of treatment with the extracts probably due to their high anti-oxidant potential but in case of standard drugs the spleen enlargement was evident and was indication of disease progression.

Disruption of joint morphology is a classical arthritic symptom that is indicative of disease stage. Joint modification results in response to bone erosion especially of cortical and trabecular bone (Sharp *et al.*, 1971). Histopathological analysis revealed modification in joint architecture after arthritis induction. A considerable difference in joint morphology was observed between normal and arthritic mice groups. Clear pannus formation, cellular infiltration and signs of bone erosion were observed in arthritic mice. The standard drug administration reduced the cellular infiltration but there was significant joint modification along with progressive bone erosion. However the extract treated groups had a different behavior, the joint architecture was preserved and signs of bone regeneration were observed. The cartilage structure was preserved in all treatment groups but it was lost in case of arthritic mice. Pattern of erosion can be a prognostic feature as the erosive changes lead to cases of disability and mortality (Scott *et al.*, 2000). Thus the histopathological analysis revealed a pattern indicative of treatment success.

Cytokines and chemokines like granulocyte-monocyte colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), Interleukin-1 (IL), IL-6 and other lymphokines induce and aggravate inflammatory responses that attract other immune cells that accumulate in the synovial cavity that leads to bone erosion and synovitis (Feldmann & Maini, 2008). This complex milieu of inflammatory cytokines and chemokines leads to the infiltration of innate and adaptive immune cells like dendritic cells, monocytes and mast cells, Th-1 cells, Th-17 cells B-cells, plasma cells and plasmablasts that initiate arthritic symptoms and lead to the prognosis of the disease. Results depicted higher IL-6 concentrations in arthritic mice model thereby leading to joint degradation as observed in histopathology. As inflammatory cytokines have

been observed to initiate cartilage degeneration through the action of MMPs (Martel-Pelletier *et al.,* 2001). IL-6 through autoantibody production and leukocyte activation generate acute phase responses such as anemia, lipid metabolism and cognitive dysfunction. In case of treatment groups leflunamide decreased IL-6 levels below normal that lead to further complications indicating a immune-compromised state whereas in case of hydroxychloroquine the IL-6 levels were significantly higher than normal. The aqueous extract treatment lowered IL-6 levels to normal whereas there was slight elevation in ethyl acetate extract treated group. The extract acted as a potent anti-inflammatory agent as it significantly suppressed IL-6 levels in comparison to the arthritic mice.

Due to synovial survival and invasiveness there is increased demand of energy that compels the metabolic shift to glycolysis thus glucose level diminish and lactate levels increase along with elevated activity of glycolytic enzymes and mitochondrial dysfunction that leads to abnormal angiogenesis, cellular infiltration and pannus formation (Biniecka *et al.,* 2016; Chang & Chao, 2011; Fearon *et al.,* 2016; Henderson *et al.,* 1979). These reduced glucose levels lead to the initiation of non-canonical autophagic pathway (Cheong *et al.,* 2011). In case of RA autophagy induction is mostly due to ER stress and proteasome inhibition (Kato *et al.,* 2014). Autophagy acts as an adaptor between cell death and survival thus is a cytoprotective and cytodestructive process in RA synovial fibroblasts (Allan & Clarke, 2009; Buckland, 2013; Kato *et al.,* 2014). Resistance to methotrexate treatment is due to increased autophagy in the cells thus making it a huge concern (Xu *et al.,* 2015). Citrullination and antigen presentation that lead to T-cell activation are regulated by autophagy (Ireland & Unanue, 2011; Scally *et al.,* 2013). Moreover studies suggest that citrullinated protein production is due to increased autophagy, thus implying that autophagy plays vital role in RA prognosis (Sorice *et al.,* 2016). Resistance to apoptosis is achieved through various mechanisms, of which increased autophagy holds prime importance as studies have revealed that rate of apoptosis is in inverse relation with LC3 and Beclin-1 (Cuda *et al.,* 2016; Gordy *et al.,* 2011). Autophagy and apoptosis are two interlinked and highly regulated processes that determine the fate of a cell. The expression analysis revealed that there was down regulation of

ULK-1 and Caspase 3 whereas the imuunohistochemistry results indicated increased LC3b expression and signs of autophagy that were observed in SEM. This was indicative of the ULK-1 independent mechanism that was in action and complicating the treatment of the disease. This is the first ever report on ULK-1 independent non-canonical pathway involved in pathogenesis of RA. The treatment groups revealed blockage of autophagic cascade and initiation of apoptosis. In case of leflunamide the ULK-1 independent non-canonical pathway remained in action with increase in Caspase 3 activity that were confirmed by the scanning electron micrographs. Whereas in case of hyrdoxycholorquine the autophagy was restored to normal as the even LC3b expression was lowered in the skeletal muscle cells. The aqueous extract treatment revealed a pattern in which Caspase 3 was up-regulated making it having a pro-apoptotic agent as there were blebs formed on the cell surface of the skeletal muscles when observed under electron microscope. However the ethyl acetate extract treatment significantly increased the expression of ULK-1 indicating a pathway shift from ULK-1 independent non-canonical pathway to canonical pathway/classical pathway. Although Caspase 3 was elevated but the pathway shift was indicative of the disruption caused by the ULK-1 independent non-canonical pathway in case of RA.

The study revealed a new pathway that is complicating the pathogenesis of RA, this depicts that there is still to be explored for proper understanding of the disease. To have better understanding other genes involved in autophagic pathway are to be explored along with characterization of plant constituents. In a nutshell, the aqueous and ethyl acetate extracts of tomoru had anti-inflammatory effect as IL-6 levels were lowered after their administration to the CIA mice model. A distinctive pattern in the expression of autophagy and apoptotic markers was observed that indicated that autophagy in RA is via ULK-1 independent non-canonical pathway. The extract administration alleviated the symptoms of RA either through induction of ULK-1 dependent pathway or apoptosis induction. This shift in autophagy and apoptotic pathway contributed to the understanding and better disease management.

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