Evaluation of anti-inflammatory and antioxidant activity of active components and potential targets of *Tracyspermum Ammi* in the treatment of Rheumatoid Arthritis

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I certify that this research work titled **Evaluation of anti-inflammatory and antioxidant activity of active components and potential targets of *Tracyspermum Ammi* in the treatment of Rheumatoid Arthritis** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred.

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DEDICATION

Every challenging work needs self-efforts as well as guidanceof elders especially those who are very close to our hearts.

My humble effort I dedicate to my sweet and loving

Father and Mother

Whose affection, love, encouragement, and prays of day andnight makes me able to get such success, the reason of what Ibecome today

Borthers

Who always pick me up on time

Whose advise, patience, faith, inspiration, and support like asoulmate, makes me the one which I appeared to the world

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Iqra Tariq

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LIST OF ACRONYMS

RA	Rheumatoid Arthritis
JRA	Juvenile rheumatoid arthritis
TNF	Tumor Necrosis Factor
ACPA	Anti-citrullinated peptides
HLA	Human leukocyte antigen
Non-HLA	Non-human leukocyte antigen
SF	Synovial Fluid
NSAIDs	Non-steroidal anti-inflammatory drugs
DMARDs	Disease-modifying anti-rheumatic drugs
MTX	Methotrexate
GI	Gastrointestinal
MCP	Monocyte chemoattractant protein
rUMP	Ribonucleotide uridine monophosphate pyrimidine
IL-1	Interleukin 1
IL-6	Interlukin 6
TNFR	Tumor necrosis factor receptor
MLKL	Mixed lineage kinase domain-like protein
NFkB	Nuclear factor k B
PKB	Protein kinase B
Casp-8	Caspase 8
MAPK	Mitogen-activated protein kinase
JAK	Janus kinases
STAT	Signal transducer and transcription activator
IRAK4	IL-1 receptor protein kinase
AP-1	Activator protein 1
MyD88	Myeloid differentiation primary response gene 88
TCM	Traditional Chinese medicine

ADME	Absorption, distribution, metabolism, and excretion
TPSA	Topological polar surface area
PDB	Protein data bank
TTD	Therapeutic Target Database
PDTD	Potential Drug Target Database
NARC	National Agricultural Research Center
OD	Optical Density
GC-MS	Gas Chromatography Mass Spectrometry
IRB	Institutional Review Board
LD50	lethal dose
WHO	World Health Organization
LFTs	Liver Function tests
RFTs	Renal Functional tests
ALT	Alanine Transaminase
ALP	Alkaline Phosphatase
NIH	National Institute of Health
ROF	Rule of five
QSAR	Quantitative Structure-Activity Relationship
BOILED	Brain or IntestinaL EstimateD
HIA	Human Intestinal Absorption
BBB	Blood-Brain Barrier
CNS	Central Nervous System
CTD	Comparative Toxicogenomics Database
STP	Swiss target prediction
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
ANOVA	Analysis of Variance
TPC	Total phenolic content

GE	Gram Equivalent
TFC	Total flavonoid content
DPPH	2,2-diphenyl-1-picrylhydrazyl
ROS	Reactive Oxygen Species
PPI	Protein-protein interaction
ALOX5	Arachidonate 5-Lipoxygenase
PTGS2	Prostaglandin-Endoperoxide Synthase 2
TYK2	Tyrosine kinase 2

ABSTRACT

Rheumatoid Arthritis is a chronic autoimmune disorder of joints typical in women and elderly people and effect 0.5% - 1% of world's population. Several genetic such as HLA genes locus and Key inflammatory process such as up and down regulation of tumor necrosis factor (TNF) alpha, interleukins, cytokines, rheumatoid factors result in RA progression. Non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying antirheumatic drugs (DMARDs) were used to treat rheumatoid arthritis but due to their adverse side effects treatment were shift from allopathy to herbal medicine because of their anti-inflammatory and antioxidant properties. The medicinal plant under study is ***Tracyspermum ammi*** (Ajwain). Phytochemical analysis was done on aqueous and methanolic extracts of T.ammi to check the medicinal components present in extracts which were future confirmed by phenolic and flavonoid content. Antioxidant activity of T.ammi were verified by DPPH assay and Hydrogen peroxide scavenging assay. Anti-inflammatory activity was assessed by Albumin denaturation assay and Collagen induced arthritic (CIA) mice model. Toxicity evaluation was done by enegide method. Based on these activities bioactive compounds were shortlisted by GCMS library that have anti-inflammatory and antioxidant properties. Pharmacological parameters of compounds related to drug design can be obtained by ADMET predictor. Target of Rheumatoid arthritis and compounds were predicted by Therapeutic target database (TTD), Comparative Toxicogenomics database (CTD), Swisstarget prediction and target hunter and their interactions were established using string and Cytoscape. By enrichment analysis pathological conditions and Common targets of rheumatoid arthritis and compounds can be predicted. Docking revealed that how much these compounds of T.ammi helps in reducing rheumatoid arthritis pathogenesis.

INTRODUCTION

1.1 Rheumatoid Arthritris

Rheumatoid arthritis (RA) is a long-term, chronic dreadful and inflammatory joint disease that affects approximately 1.0% of the total population mainly adults worldwide. Rheumatoid arthritis is a systemic and inflammatory autoimmune disorder leading to swelling and pain in small and large joints of hands and feet, typically presenting between the ages of 30 and 50 years typical in women and elderly people (Scott et al., 2010). RA generally exhibits inflammation of affected joints being stiff, painful, warm, and swollen, specifically on walking in a break of day or after perpetuating inactivity. The joints are frequently affected in a relatively uniform fashion. As the disease progresses the provocative activity manifests tendon tethering, destruction, and erosion of the joint lining, which compromise the extent of movement and promote deformity (Yang et al., 2013).

Communal symptoms of rheumatoid arthritis comprise morning stiffness of the exaggerated joints for more than 35 minutes, fever, fatigue, tender joints, weight loss, warm and swollen, and also profound diffuse soreness and inflammation in the pericardium, lungs, sclera and pleura, nodular lesions, frequently in subcutaneous tissue. The inception of RA is usually from ages 36-60 with exacerbation and remission. It also badly affect young children even before age 15 and is described as juvenile rheumatoid arthritis (JRA), which is analogous to RA with the exception of that rheumatoid factor (Chaudhari et al., 2016).

Pathological variations in RA are infiltration of inflammatory cells, hyperplasia of the synovial membrane, and neovascularization, which causes articular destruction and cartilage erosion (Ngoc et al., 2005).

1.2. Epidemiology

In Pakistan, the greatest prevalence (8/1,000) was seen in the prosperous metropolitan community (HAMEED & GIBSON, 1996). Clinical features observed in Pakistani hospitals disclosed an average time of 69 minutes patient grumbles of daybreak stiffness in joints. 7% of cases were discovered with the nodular disease. Extra-articular characteristics are noticed in 14%

of patients. Commensurable arthritis was observed in all the patients in India, and 87.8% of cases were found of morning stiffness in the population (Akhter et al., 2011).

One study showed point prevalence of RA reported from Karachi was elevated at 26.9% which are largely females but no study investigated the impact of this ailment on household and family job performance of rheumatoid arthritis-affected women in Pakistan. Mild functional disability (51.66%) was seen in RA patients, almost half (46.8%) had comorbidities. The fifth proportion of RA patients had dyslipidemia as a comorbidity (16.7%) and a greater cardiovascular risk score as a modifiable risk factor. Undiagnosed depression (58.32%) and low bone mineral density (40.56%) were reported in RA patients (AA et al., 2019).

According to a study in Ontario, Canada in 2010, Widdifield et al. 2014 reported that overall, a 0.06% increase in discrete cases per year depicts that RA is growing at an alarming rate. They reported the unsophisticated number of individual cases detected each year was 5,524 patients in 1996 and 6,397 patients in 2010 and the prevalence of RA in Ontario was 0.96% (Widdifield et al., 2014).

One study reported the pervasiveness of RA in different countries as 0.3% in Italy, 1.09% in the USA, Pima Indians, and Chippewa Indians in Arizona and have the occurrence of 6.72% and 5.41% respectively. However, people residing in emerging countries are noticed to have prevalence as low point as 0.0126% in rural Africa, 0.172% in the Philippines, and 0.21% in China (Akhter et al., 2011).

1.3 Pathogenesis

Rheumatoid arthritis is prime deemed a clinical functional disorder straddling many disease subcategories. These distinct divisions necessitate numerous provocative cell cascades, which all edge towards a definitive familiar pathway through which persistent continual synovial infl ammation and correlated associated damage to trabecular cartilage and fundamental bone are existing (Mil & Huizinga, 2008). The pathophysiology flow chart is seen in figure 1.1

1.3.1 Inflammation

Top significant incendiary cascade entails overexpression and overproduction of TNF. This path leads to denaturation of joint and cartilage and synovial fluid inflammation. Excess of

TNF has numerous causes, incorporating interactions between B and T lymphocytes, macrophages, and synoviocytes. This process drives the procurement of many cytokines such as interleukin 6 and 1, which also propels unrelenting joint destruction and inflammation (Choy et al., 2002).

1.3.2 Synovial and cartilage cells

The prevailing cell populations in joints damaged by RA are cartilage and synovial cells. Macrophage-like and fibroblast-like synoviocytes are the two divisions of synovial cells. The procurement of chemotactic cytokines is considered to be directed principally by macrophageial synoviocytes. Fibroblastic synoviocytes illustrate atypical conduct in RA. In investigational prototypes, co-embedding of cartilage with fibroblastic synoviocytes which drives fibroblasts infecting cartilage that associates together with synovial joint deformation and destruction (Tolboom et al., 2005).

1.3.3 Autoantibodies

Rheumatoid factor is the archetypal autoantibody in RA. IgA and IgM RA factors are vital infective indicators targeted counter to IgG Fc fragment. Further types of antibodies are those which targeted contrary to citrullinated peptides (ACPA). Rheumatoid factor is not positive for all of the ACPA-positive patients, ACPA appears more particular and susceptible for identification and seems to be efficient prognosticators of mediocre aspects such as radical joint denaturation and devastation. The purpose of proceeding research is to pinpoint antibody specialities appropriate for diverse patients' subgroups and illness phases. 60–85% of entities with RA have ACPA, rheumatoid factor, or in cooperation (Oosterhout et al., 2008).

Conclusions of clinical examinations depict that incidents with RA and together ACPA and RA factor vary between individuals described as autoantibody-Positive disease. For example, according to history, incidents having positive ACPA RA have higher inflamation caused by lymphocytes in synovial joint and tissue, whereas individuals with negative ACPA RA have higher fibrosis content and expanded of the synovial stratum thickness. Positive ACPA disease is correlated with intensified joint denaturation and lesser diminution rates (Mil et al., 2005).

1.3.4 Genetics

50% of the risk of emerging rheumatoid arthritis is associated with genetic factors which involve HLA and non-HLA genes. Genome-wide association studies describe that HLA-DR genes are mostly entailed in RA pathophysiology which acts as predisposing peptide factors responsible for antigen presentation to cells in adaptive immune systems and demonstrates association to the production of elevated levels of rheumatoid factors and ACPA (FIRESTEIN, 2018). The most ordinary non-HLA genes that are associated with disease progression include PTPN22 involves in signaling through the T-cell receptor. Many other enzymes such as matrix metalloproteinases entail joint destruction and inflammation and their activation is highly negotiated by proinflammatory cytokines. The most important MMP-9 level leads to a progression of joint denaturation and destruction in synovial fluid (SF) and blood plasma (BP) of patients with RA (Karami et al., 2019).

Other confirmed and principle susceptible loci which had a risk for RA are; IL2RB, BLK TRAF1, FCRL3, CRP, IL2RA, STAT4, IL-4 receptor, CTLA4, IL6ST, IRF5, CCR6, C5orf30, CD40, SPAG16, IL6ST, CCR5, FCRL3, CCL21, TNFAIP3, ZFP36L1, PTGER4, TNF-α, IL-4, DKK-1, Granzyme B, TNFAIP3, FOXO3, IL15, SPP-1, TNFRSF11B, KIR, TYK2, RUNX1, and many others (Karami et al., 2019) (Koga et al., 2021).

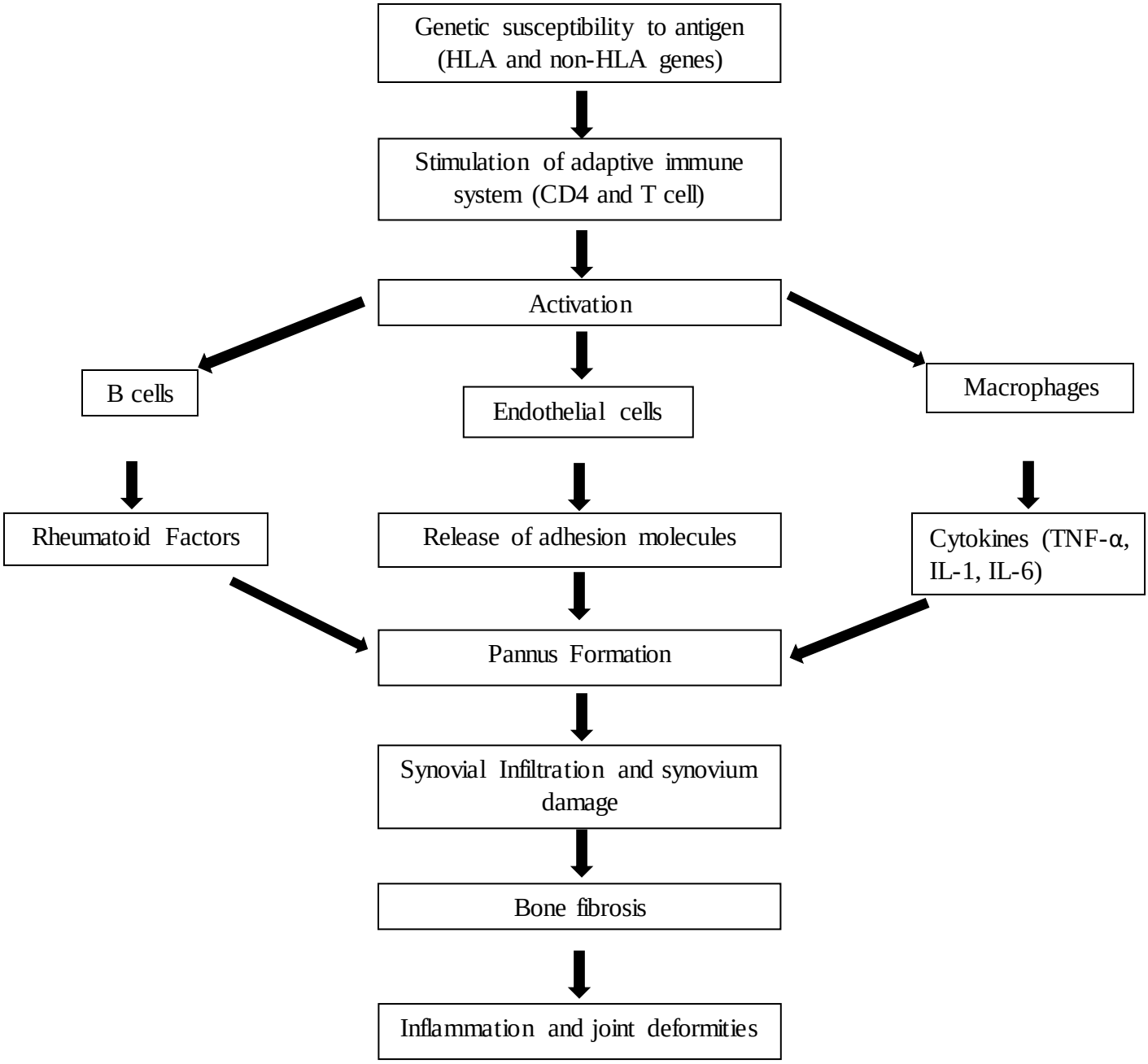


Figure 1.1: Possible molecular mechanisms of Rheumatoid Arthritis progression.

1.3.5 Environmental risk factors

Recent findings have enhanced our awareness of environmental natural contacts that increase susceptibility for RA. The topmost identified environmental risk factor involved in RA pathogenesis is smoking and going to increase with the amount and extent of cigarette use (Liao

et al., 2009). Another key risk factor comprises alcohol intake which may lessen the probability for RA pathogenesis. On research aim to assessing persons who intake liquor and people who did not consumed, perssons who drank liquor had bare minimum danger of emerging positive ACPA RA (Pedersen et al., 2006). High birth weight (>4.1 kg) stayed susceptible by a three-fold elevate threat of arthritis in a study led in Sweden. One study uncovered that breastfeeding with a farseeing period of greater than 12 months heve lesser danger of RA progression (Jacobsson et al., 2003).

1.4 Clinical implications and diagnosis

Before the 1990s, the diagnosis of RA was escorted by serious consequences, which predictably culminated in joint destruction, and astonishing disability in bones (Abbasi et al., 2019). Diagnosis of rheumatoid arthritis is very difficult in its early phases because the initial indications and symptoms imitate several other diseases as well. There is no physical testing and blood test to validate the identification. Throughout the physical examination, check swollen joints, warmth and redness, muscle strength and reflexes.

1.4.1 Disease Activity

It involves arena of bone and cartilage erosion and joint denaturation and impairment generally depend on inflammation which involves:

1.4.1.1 Core Measures

It mainly includes tender and swollen joint count which is a doctor-based global appraisal. Laboratory procedures incorporate erythrocyte C-reactive protein, sedimentation rate, or together. Persistent methods assess soreness, worldwide estimation, and debility. The health assessment questionnaire (HAQ) estimates debility (Dougados et al., 2007).

1.4.1.2 Combined Indices

Indices integrate individual appraisals. The disease activity score 28 (DAS28) combines 28 tender and 28 swollen joints (knees, hands, and arms), erythrocyte sedimentation rate, and the patient's global assessment to reveal the patient's existing status (Aletaha & Smolen, 2009). Standard disease joint count focuses on 28 joints in the hands, upper limbs, and knees; joints in

the feet even though essential, have being excluded. Several specialists would prefer extended 66 and 68 joint count up including the feet (Dougados et al., 2007).

1.4.2 Extra-articular disease

It involves subcutaneous nodules, pulmonary consequences include pleural effusion, pulmonary nodules, fibrosing alveolitis. Ocular consequences include keratoconjunctivitis Sicca, scleritis, episcleritis. Cardiac consequences include pericarditis, valvular heart disease, pericardial effusion, transmission imperfections. Neural consequences include nerve entrapment, peripheral neuropathy, cervical myopathy. Cutaneous consequences include palmar erythema, vasculitic rashes, pyoderma gangrenosum, leg ulceration (Young & Koduri, 2007).

1.4.3 Comorbidities

It involves cardiovascular maladies like myocardial infarction, stroke, heart failure, hypertension, peripheral vascular disease. Other comorbidities consist of lymphoma, lung and skin cancer, lymphoproliferative diseases, depression, bacterial infections, gastrointestinal diseases, psoriasis, osteoporosis, and various renal diseases. (Liao et al., 2009).

1.5 Treatment

The major objective of treatment is concentrated on the way to reducing the disease pathology or inflamed situation with overall remission, if possible, by decrease in joint denaturation and destruction results in developing healthy the bodily fitness and wellbeing.

1.5.1 First Line Management

The fundamental goals of first-line administration are to relieve pain and reduced sourness and inflammation which primarily entails NSAIDS (non-steroidal anti-inflammatory drugs) and Corticosteroids explained in table 1.1 (Bullock et al., 2018) (Abbasi et al., 2019).

1.5.2 Second Line management

The whole aim of second-line medication is to stimulate retardation by slackening or stopping the disease progression inclusive of joint deformity and destruction. These treatments possess DMARDs (Disease-modifying anti-rheumatic drugs) which are liberated as slow therapy

for the reason that they take over weeks or months for gaining helpful effectively and explained in table 1.2 (Bullock et al., 2018)(Abbasi et al., 2019).

Table 1.1: First-line treatment of Rheumatoid Arthritis.

Drug Type	Names	Role	Side effects
Non-steroidal anti-inflammatory drugs (NSAIDS)	**Acetylsalicylate** (Aspirin), **Naproxen** (Naprosyn), **Ibuprofen** (Advil and Motrin), **diclofenac**, **Etodolac** (Lodine)	Works by inhibiting cyclooxygenase to preclude production of prostacyclin and prostaglandins, thromboxane.	ulcers, abdominal pain, Nausea, gastrointestinal (GI) bleeding
Corticosteroids	**Glucocorticoids**	works by preventing phospholipid release and decreasing actions of eosinophils, therefore decreasing inflammation	weight gain, diabetes, bone thinning, immunosuppression

Table 1.2: Second-Line Treatment of rheumatoid arthritis.

Drug Type	Names	Role	Side effects
DMARDs	**Methotrexate (MTX)**	immunosuppressive drug	liver problems, cirrhosis, and bone marrow deterioration
	Hydroxychloroqyine (Plaquenil)	minimum emission of monocytic cytokines	the gastrointestinal tract, skin, and CNS

	Sulfasalazine (Azulfidine)	decrease emmision of monocyte chemoattractant protein (MCP) and interleukin 8 (IL-8)	gastrointestinal and central nervous system
	Aurothioglucose (Solganal), **Auranofin** (Ridaura), **Gold sodium thiomalate** (Myochrysine), **D-penicillamine** (Depen, Cuprimine)	Immunosuppression	Not effective as methotrexate
	Azathioprine (Imuran), **Cyclophosphamide** (Cytoxan), **Chlorambucil** (Leukeran), and **Cyclosporine** (Sandimmune),	Reducing the accumulation of toxic elements in joints	blood problems, muscle aches, and pain

1.5.3 Newer Medications

New therapeutic approaches includes biological agents drugs that focus on proximal impacts on the immune system activation for T-cell subcategories procurement and several inhibitors explained in table 1.3 (Bullock et al., 2018) (Abbasi et al., 2019).

Table 1.3: Current medications in the treatment of Rheumatoid Arthritis.

Drug Type	Names	Role	Side effects
Biological agents	**Leflunomide**	constrains the production of ribonucleotide uridine monophosphate pyrimidine (rUMP)	hypertension, gastrointestinal upset, leukopenia, liver damage, neuropathy, rash bone marrow damage and interstitial lung disease
	Etanercept (Enbrel), **Infliximab**	TNF-inhibitors	contraindicated in patients with congestive heart failure, insomnia, low blood pressure.

(Remicade), **Adalimumab** (Humira),		
Anakinra (Kineret)	Interacting with interleukin 1 (IL-1)	a lower response rate
Rituximab (Rituxan)	Depletes defective B cells, Treat RA complications such as vasculitis and cryoglobulinemia	Insomnia, Peripheral edema, headache, decreased appetite, increased triglyceride level, muscle aches and pain, fever, abdominal pain, back pain
Abatacept (Orencia)	blocking T cell activation	Hypertension, infection
Tocilizumab (Actemra)	blocking interleukin 6 (IL-6)	gastrointestinal upset, liver damage, CNS disorders
Tofacitinib (Xeljanz)	JAK inhibitor	serum sickness, arterial and venous blood clot, thyroiditis

1.5.4 Herbal medicine

Due to devastating side effects reported by the practice of allopathic medicines treatment of rheumatoid arthritis shifted to herbal medicine provided by medicinal plants. There are many plants reported that have anti-rheumatic, anti-inflammatory, and antiarthritic potential that causes remission of arthritic illness. Botanical medicines have been used as alternative approach for treating various diseases as they are considered safe, effective, and have been used in various traditions to treat patients for many years. These plants include *Harpagophytum procumbens, Bauhinia variegate, Acalypha indica,* and many more. (Nimesh, 2018). A wide range of phytochemicals containing phenolic acids, triterpene, glycosides, triterpenoid, diterpene saponin, phenylpropanoid ester, and flavonoids has been recognized and proven to be liable for the biological effects of the herbs. Insight into the mechanisms of action of the herbs may provide new treatment opportunities for RA patients (Yang et al., 2013). In this study ***Trachyspermum ammi, a*** common name (**Ajwain**) a medicinal plant is used for the treatment purpose of RA. Gas

Chromatography-Mass spectrometry (GCMS) and phytochemical analysis revealed druglike compounds used as a therapeutic agent for the remission of arthritic illness in near future.

1.6 Network pharmacology

This one is commonly understood that discovery of drug frequently necessitates a systems-level Poly pharmacology approach to deal with challenges such as paucity of efficiency and developing resistance of specific-pursued constituents. Network pharmacology methodologies are progressively attended to established and employed to discover modern therapeutic restorative opportunities and to repurpose authorized drugs. Nevertheless, these latest innovations have been comparatively transformed into the sphere of biological inventions gradually. Now, we claim that a network pharmacology approach ought to assist an efficient plotting of the still unfamiliar functional area of biological agents, therefore offering a efficient method to expand the druggable area of targeted proteins associated with a variety of intricate and complicated diseases (Kibble et al., 2015).

The current study concentrates primarily on in-silico approaches using in-vivo and in-vitro transcriptomic drug response profiles by recent developmental and conventional tools of network pharmacology.

1.7 Objectives

The aim and objective of this study is to investigate potential therapeutic compounds present in *Trachyspermum Ammi* in the treatment of Rheumatoid Arthritis.

Review of literature

2.1 Ethnobotany

Rheumatoid arthritis is an enduring, autoimmune, and inflammatory disease that affects synovial joint denaturation. Indicative pathogenicity of RA is not entirely identified which leads to severe cartilage and bone destruction and abnormalities in large and small bones of hands and feet. Bone denaturation and destruction lead to synovial inflammation which affects walking and obstructs many physical activities perform. Physicians recommend countless allopathic medicines to repossess from this disease like non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids that include aspirin and ibuprofen type painkillers and disease-modifying anti-rheumatic drugs (DMARDS) which includes metformin, methotrexate. Other conventional treatment methods include biologics that have rituximab and tofacitinib type drugs. But these traditional allopathic therapies are very expensive and takes years to get the recovery and have devastative side effects like intestinal problems, blood, blood pressure problems, and protein loss that can never be treated by these conventional treatment methods. Uce Ozkol et al in 2014 reported the incident of mucositis in 32 years old women due to methotrexate (Ozkol et al., 2015). So, to lessen illness and to manage disease outcomes, treatment mechanism shifts from allopathy to herbal, and scientists had done substantial work to found medicinal plants that have anti-arthritic, anti-rheumatic, and anti-inflammatory activities. Many studies reported that herbal medicine is less harmful than allopathic drugs, had immunomodulatory effects, and could be used for the reduction in inflammation and immune dysfunctions of RA patients (Wang et al., 2021) (Lü et al., 2015)

2.2 Inflammatory pathways in Rheumatoid arthritis

Many environmental and genetic predisposing factors cause the production of certain metabolites which inturns activate the adaptive immune system which regulates certain chemotactic pathways involves in the progression and pathogenesis of Rheumatoid Arthritis. This induces lymphocytes (Tand B cell) and endothelial activation, chemokine, and cytokine activation results in angiogenesis of synovial tissues and fluid lead to inflammation.

2.2.1 TNF mediated Signaling pathway

TNF (Tumor necrosis factor) is a profound cytokine that causes inflammation in many diseases one is Rheumatoid Arthritis (fig 2.1). TNF is a homotrimer bind with tumor necrosis factor receptor (TNFR) which is divided into two transmembrane receptor proteins TNFR1 and TNFR2. After binding several protein complexes are activated which mainly involves TNFR1 associated death domain protein (TRADD) and TNFR1 & 2 associated factor 2 complex (TRAF2). TNFR1 complex activates Mixed lineage kinase domain-like protein (MLKL) induce inflammation and necrosis and Caspase 8 (Casp-8) induce apoptosis of synovial tissues. TNFR2 and TNFR2 both significantly mediates activation of nuclear factor k B (NFkB) and mitogen-activated protein kinase (MAPK) and protein kinase B (PKB) which induce synovial tissue degradation, inflammation, abnormal cell proliferation, and halters cells survival (Noack & Miossec, 2017.)

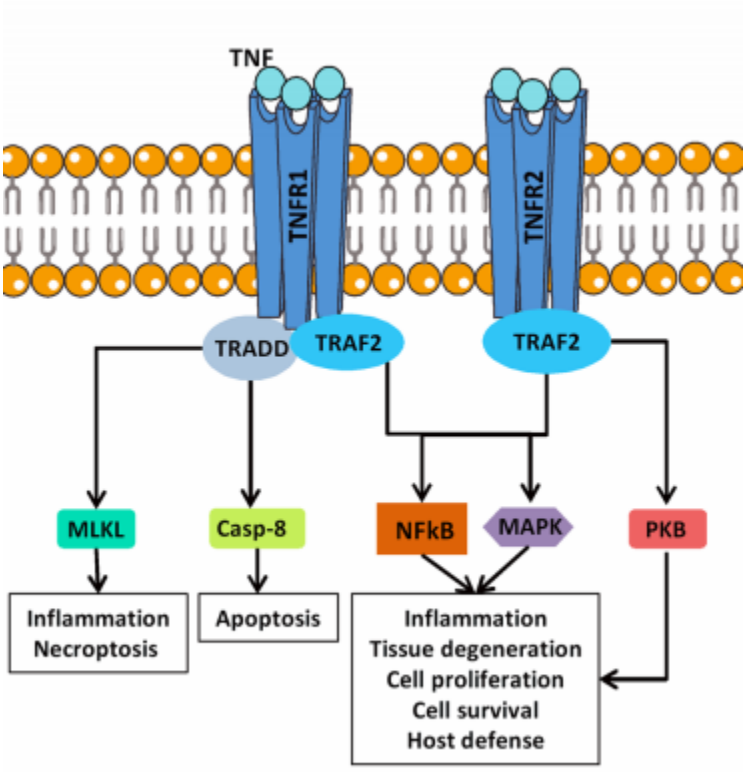


Figure 2.1: TNF mediated signaling cascade in RA (Noack & Miossec, 2017.).

2.2.2 IL-6-mediated signaling pathway

Interleukin 6 is the inflammatory cytokine produced by inflamed immune cells and its expression is mainly restricted to megakaryocytes and lymphocytes but gp130 has a ubiquitous nature. IL-6 plays two distinctive cascade mechanism one is classical and the other is trans-signaling. In classic signaling, two subunits of IL-6R are used which include IL-6R and gp130 mediated by membrane-bound IL-6R subunit (mIL-6R). Trans-signaling requires a soluble form of the IL-6R subunit (sIL-6R). Both these signaling receptors mediate the activation of JAK proteins which in turn activates STAT proteins and MAPK proteins induce chronic inflammation, TH17 differentiation followed by cell growth (fig 2.2)

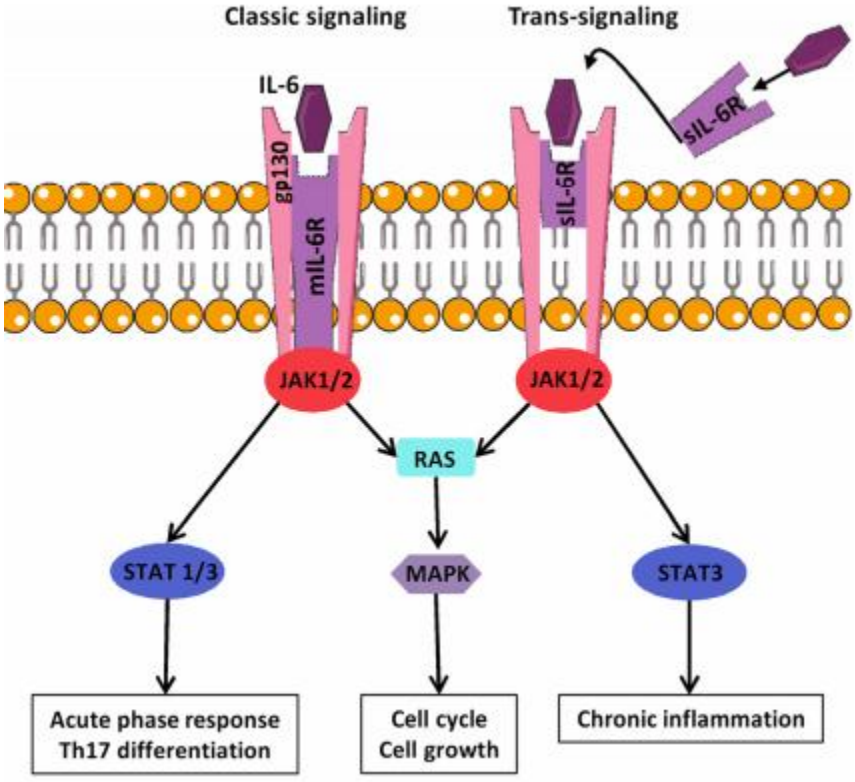


Figure 2.2: IL-6 mediated signaling pathway. (Noack & Miossec, 2017.)

2.2.3 IL-1 mediated signaling pathway

IL-1 is a proinflammatory cytokine first identified in RA pathogenesis mainly secreted by macrophages, dendritic cells, and monocytes. IL-1R in combination with IL-1R accessory proteins (IL-IR1AcP) form active IL-1R1 protein which binds to myeloid differentiation primary response gene 88 (MyD88) after binding of the receptor to the ligand IL-1. MyD88 then activates

the IL-1 receptor protein kinase (IRAK4). This stimulates the polymerization and activation of TRAF6 which in turn activates NFkB and activator protein 1(AP-1). This cascade influence host defense, osteoclast maturation, cell proliferation followed by cell survival. (Fig 2.3).

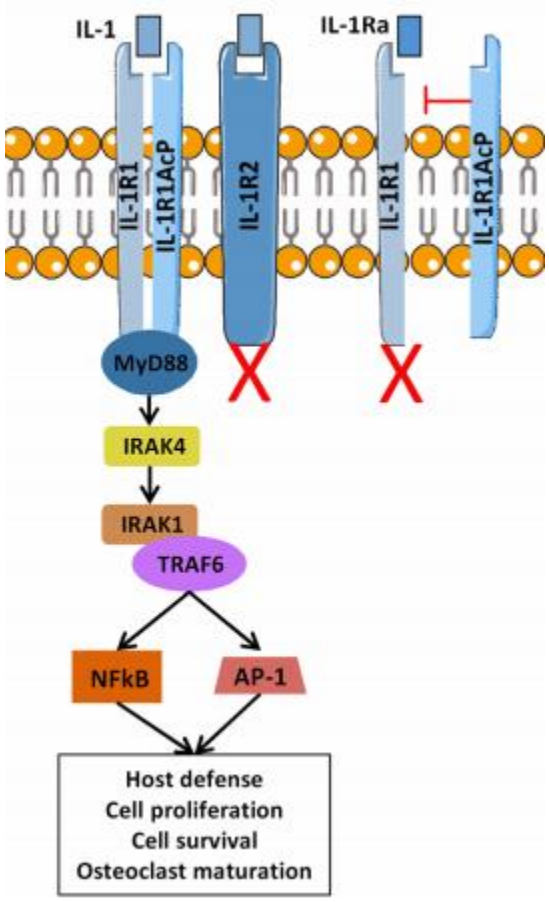


Figure 2.3: IL-1 mediated signaling pathway. (Noack & Miossec, 2017.)

2.2.4 IL-17 mediated signaling pathway

IL-17 is a pleiotropic cytokine that is secreted by T cells mainly TH-17. IL-17 binds to IL-17RA results in the recruitment of IL-17RC forming a complex and activates the ubiquitin ligase Act-1 by the signaling domain SEF/ IL-17R (SEFIR). This complex then recruits TRAF6 leads to the activation of NFkB, MAPK, and PI3K cause inflammation, cartilage damage and bone erosion. (Figure 2.4).

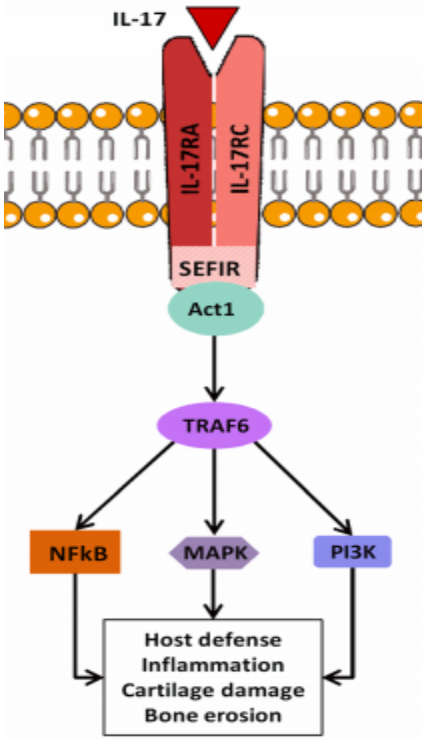


Figure 2.4: IL-17 mediated signaling pathway. (Noack & Miossec, 2017.)

2.3 Rheumatoid Arthritis medicinal plants

Herbal therapy is a persuasive treatment for the disease illness as it offers a safe and efficient standpoint for the disease progression. To date, many plants founds have pharmacological properties to treat RA which are progressively used as herbal medicine and most common in Traditional Chinese medicine (TCM) many of them are underneath scientific scrutiny (table 2.1) (Pandey et al., 2018).

Table 2.1: Most common TCM herbal Interventions in the treatment management of Rheumatoid arthritis.

Sr no	Botanical name	Common Name	Family	Reference
1	Aloe barbadensis	Aloe vera	Asphodelaceae	(Tiwari & Sharma, 2017a)
2	Andrographis paniculata	King of bitter	Acanthaceae	(Yan et al., n.d.)
3	Borago officinalis	Starflower	Boraginaceae	(Alamgeer et al.,

				2017)
4	Cannabis sativum	Marijuana	Urticaceae	(Gonen & Amital, 2020)
5	Celastrus paniculatus	Black oil	Celastraceae	(Kulkarni et al., 2015)
6	Cinnamomum Verum	Dalchini	Lauraceae	(Vetal et al., 2013)
7	Coriander sativum	Dhania	Umbelliferae	(Nair et al., 2012)
8	Curcuma longathe	Turmeric	Zingiberaceae	(Taty Anna et al., 2011)
9	Glycyrrhiza glabra	Liquorice	Leguminosae	(Gonen & Amital, 2020)
10	Moringa oleifera	-	Moringaceae	(Fard et al., 2015)
11	Nyctanthes arbortristis	Night Jasmine	Oleaceae	(Tiwari & Sharma, 2017)
12	Piper nigrum.	Black pepper	Piperaceae	(Koga et al., 2021)
13	Punica granatum Linn	Pomegranate	Lythraceae	(Bang et al., 2009)
14	Ribes Orientale	Ghonashatooh	Grossulariaceae	(Shukla et al., 2008)
15	Swertia chirayita	Chirayita	Gentianaceae	(Vyas et al., 2019)
16	Withania somnifera	Indian ginseng	Solanaceae	(Khan et al., 2018)

2.4 *Trachyspermum Ammi* -A miracle plant

Trachyspermum Ammi is generally known as **Ajwain** related to the family **Apiaceae** is originated from Sanskrit ajamodaor ajamodika. It is annual herbaceous and aromatic plants usually have gray-brown seeds (Rajeshwari et al., 2011) in figure 2.5. Ajwain is extensively distributed and nurtured in many regions like India, Pakistan, Afghanistan, Bangladesh, and many more usually grown in October-November and harvest in May-June, flowers, and fruit-bearing on the period of January -April (Zarshenas et al., 2014). T.ammi is approximately 90-93cm tall and encompassing of more than 260 genera and species has entitled by many vernacular names such as Bishop's weed (English), dipyaka (Sanskrit), lodhar (Punjabi), yavan

and javan (Bengali), Kath (Kashmiri), Onva (Marathi), omam (Tamil), ajowan (Portuguese), omu (Telegu), chilan (Thai), misir anason (Turkish) (Rajeshwari et al., 2011)(Kumar, 2017).

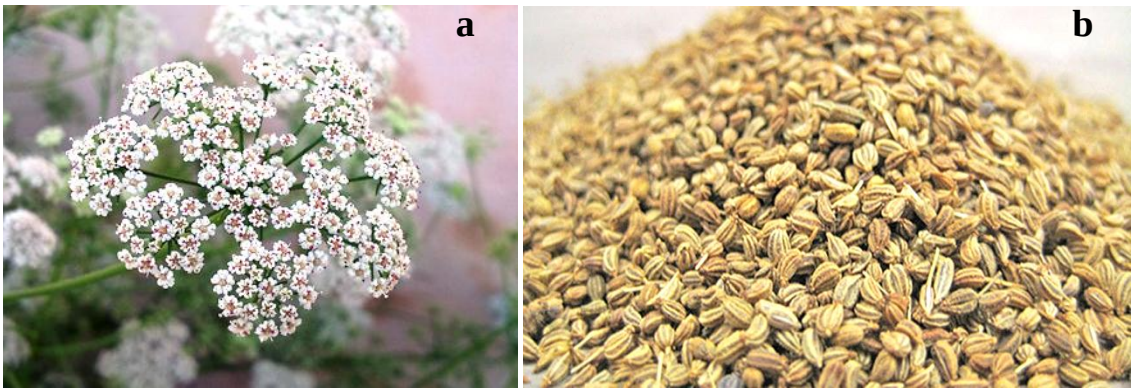


Figure 2.5: *Trachyspermum Ammi* (Ajwain) flowers (a) and seeds (b). (Noack & Miossec, 2017.)

The essential oil variety of T.ammi seeds is 2.5-5% which comprises many nutritive components which accounts for approximately 96 gram percent of the overall amount. Essential oil mainly involves carbohydrates (38.7 g%), Fibers (12 g%), moisture (8.9 g%), Proteins (15.4 g%), fats (18.1 g%), Minerals (7.1 g%), calcium (1.4 g%), phosphorus (0.4 g%), Iron (14.5 mg%) (Rajeshwari et al., 2011), (Kumar, 2017), (Zarshenas et al., 2014).

T.ammi essential oil indicated the existence of 26 classified components which account for 97.3% of the total amount. Supercritical fluid (CO_2) only contains thymol (39.1%) originate as a main constituent along with p-cymene (30.8%), Hydro distilled oil contains γ-terpinene (30.8 %), thymol (49.0%), limonene (0.7%), myrcene (0.8%), β-pinene (2.1%), and p-cymene (15.7%) however acetone extract of T.ammi exhibited the occurrence of 18 recognized constituents which report 68.8% of the overall quantity. The main constituents of acetone extracts of T.ammi were thymol (39.1%) fo oleic acid (10.4%), linoleic acid (9.6%), (-terpinene (2.6%), p-cymene (1.6%), palmitic acid (1.6%) and xylene (0.1%) (Gurdip Singh et al., 2004) (Chahal et al., 2017).

2.5 Taxonomical Distribution

Kingdom : Plantae

Division : Magnoliophyta

Order : Apiales

Class : Magnoliopsida

Family : Apiaceae

Genus : Trachyspermum

Specie : Ammi

Reference : (Bairwa et al., 2012)

2.6 Ethnopharmacology of *Trachyspermum Ammi*

Trachyspermum ammi gets widespread pharmaceutical applications being treated for many disorders for gastrointestinal problems like abdominal tumors, abdominal pains, piles, polyuria, bronchitis, asthma, galactagogue, toothache, and common cold (Rajeshwari et al., 2011). Medicinally T.ammi is identified to be effective against many bacterial and viral infections and has pharmacological properties like antinociceptive, antimicrobial, antioxidant, antifungal, cytotoxic, antispasmodic, hypolipidemic, anti-hyperactive, diuretic, antitussive, nematocidal, antirheumatic, anthelmintic, and ant filarial (Dubey & Kashyap, 2015).

2.6.1 Insecticidal activity

Secondary metabolites of plants play a significant function in the plant and insect interfaces. Some composites obtained from parts of plants possess insecticidal properties. The oil obtained through the seeds of T.ammi showed insecticidal mechanism counter to *Callosobruchus Chinensis* in the oviposition stage besides egg hatching and evolving inhibitory properties (MK, 2008). The alcoholic extract of T. ammi seeds was illustrated as effective towards larval and pupal stages of Aedes aegypti and estimated as an eco-approachable remedy. The death percentage of the pupa was three times lesser than the larva stage (Chahal et al., 2017).

2.6.2 Antidiarrheal activity

Anti-dysenteric and antidiarrheal attributes of medicinal plants were uncovered to be due to the existence of flavonoids, saponins, triterpenes sterols, reducing sugars, tannins, and alkaloids. The phytochemical findings on T. ammi kernels have reported the presence of tannins, sterols, flavonoids, and saponins. Thus, the antidiarrheal property could be due to these chemical

elements. Flavonoids possess antidiarrheal activity, which may hamper hydro electrolytic secretions and intestinal motility which are found to be altered in diarrhea (Perianayagam et al., 2005).

2.6.3 Antibacterial activity

Acetone and ethanol extract of T.ammi seeds had antibacterial properties counter to two Gram-negative food degeneration bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Acetone extract of T.ammi seeds acquired greater efficacy counter to P. aeruginosa although ethanol extract possessed greater efficacy counter to E. coli (Usha et al., 2012). Methanol extract of T.ammi seeds exhibited antibacterial properties compared to certain species of bacteria like Staphylococcus aureus, S. epidermidis, P. aeruginosa, Bordetella bronchiseptica Klebsiella pneumonia, and E. coli (Bonjar, 2004). However, aqueous and acetone extract of T.ammi seeds displayed antibacterial efficacy against Enterococcus faecalis, E. coli, K. pneumonia, S. Typhimurium, P. aeruginosa, Salmonella typhi, Shigella flexneri, and S. aureus employing agar diffusion assay (Arora & Kaur, 2009).

2.6.4 Antioxidant activity

T. ammi is abundant in minerals and vitamins; it is likewise intense in health stimulating nutrients for instance flavonoids and carotenoids (lutein and βcarotene) that illustrate significant shield against antioxidants. The antioxidant capability of an amalgam of T. ammi seed extricate have being investigated by assays; the hydrogen peroxide (H2O2) and nitric oxide free radical scavenging assay. Findings reported that T.ammi extricate proposed a substantial influence on impeding hydrogen peroxide and nitric oxide free radical. (Kumar Bajpai et al., 2015.).

Ethanol extract of T. ammi essential oil exhibited a robust antioxidant potential determined by certain scavenging models involving DPPH, hydroxyl radical scavenging assay, superoxide nitric oxide, and lipid peroxidation capability in the bovine serum and brain extract. Ethanol extricate of T. ammi essential oil reduced 75.42% of DPPH radicals and also possessed a strong repressive activity on rummaging nitric oxide, hydroxyl, and superoxide radicals by 67.23, 64.49%, and 63.32 correspondingly. Every bit of these antioxidant activities verified the pharmacologic effectiveness of T.ammi as a probable resource of organic antioxidants (Pathak et al., 2014).

2.6.5 Antifungal activity

T.ammi seed oil also possessed a wide variety of toxic actions of fungi against, A. flavus, A. niger, A. ochraceus, P. viridicatum, P. citrium, Cheilomenes lunata, P. madriti, and total region reticence was achieved at a 5 µl dosage of the essential oil (Thangam & Dhananjayan, 2021). T.ammi seeds inhibited the expansion of all test fungi by 73-90%. The antifungal consequence of T.ammi seed essential oil on Cochlostyla ovoidea and A. Niger was also investigated and a bare minimum inhibitory concentration was predicted at 5000µg/ml. Phenolic constituents, such as carvacol and thymol, are found to be either bacteriostatic or bactericidal agents differing on the concentration utilized (Dwivedi & Singh, 1998.).

2.7 GC-MS of Trachyspermum Ammi

The essential oil chemotype of T.ammi was detected by gas chromatography-mass spectrometry (GC-MS) analysis. Soltani Howyzeh et al., 2018 reported 15 essential oil compounds detected by GCMS. According to this analysis yield of essential oil is 5.9% which is greater than the previously reported yield of 2-5%. Fifteen reported compounds are 1,8-Cineole, α-Thujene, Myrcene, α-Pinene, α-Terpinene, δ-3-carene, β-Pinene, β-Phellandrene, ρ-cymene, Terpinolene, γ-Terpinen, α-Terpineol, Thymol, Terpinene-4- ol, and Carvacrol.

Another study investigated pharmacological compounds in T.ammi seed extract are 6-octadecenoic acid, methyl ester, 3,5-dimethylanisole (83.19%), 2-cyclohexyl-2,5-cyclohexadiene-1,4-dione, 4-oxime (3.01%) and (Z)-, 7-octadecenoic acid, methyl ester (7.42%), (Abdullah et al., 2020).

2.8 Network Pharmacology

A leading paradigm in drug discovery is the notion of designing outstanding ligands to act primarily on entities' drug targets. This idea is not man-made but reproduced precisely from nature. Nature has developed divergent strategies to regulate biological practices either by selectively pursuing biological macromolecules or by establishing molecular promiscuousness or Poly pharmacology define as one molecule combines with too many targets. Network pharmacology underscored the significance of examining ligand-target networks to evaluate botanical drugs. (Vlietinck et al., 1998). Network pharmacology, centered on the concept of

systems biology, is a modern discipline that assesses the biological network and monitors out the nodes of particular interest, intending to design poly-target drug molecules. It accentuates increasing drug effectiveness and reducing devastative effects through the multiple adjustments of the signaling cascades (Hao & Xiao, 2014).

Intriguingly, the network pharmacology theory certainly connects back up to pharmacognosy. The perception of employing concoctions of plants ('botanical drugs') for treatment is very famous in conventional plant therapy. For example, Traditional Chinese Medicine (TCM) formulae often comprise of permutations of botanical herbs where manifold active plant components might hypothetically aim many targets/pathways (Kibble et al., 2015). One research brought through concentrating largely on cheminformatics and virtual screening (VS) techniques, counting pharmacophore hunt, inverse docking, molecular docking, and QSAR modeling employing chemical signifiers and fingerprints to stretch a twist in network pharmacology by merging them with drug target relationship along with their pathway information. (Lagunin et al., 2014).

A study was carried out in North Africa in which components of North African plants were retrieved from a community database and ADME screening was employed for purification of constituents using Qikprop software. STITCH database was sourced for predicting the plant components targeted proteins/genes, Uniprot database was exploited for spotting genes associated with cancer. Compound-target gene (C-T), compound-pathway (C-P), and plant-compound-target gene (P-C-T) networks were established using Cytoscape to interpret the anti-cancer mode of action of the plants. GO enrichment analysis and the KEGG pathway were done to explore the molecular processes and pathways correlated to cancer (Shawky, 2019).

2.9 Drug Likeliness properties

The ADME (absorption, distribution, metabolism, and excretion) approach was applied for the purification of components formulated on drug-likeness features. The evaluation of ADME properties is essential in drug development and easily assess through the swissadme web tool. Nevertheless, in silico prediction of ADME properties must be gradual to make up transformation from developmental drug to research of biological product (Kibble et al., 2015). Drug-likeness filtering was based on Lipinski's rule of five (Lipinski et al., 1997). Drug-like

properties can also be filtered by Ghose (Amgen) (Arup K. Ghose et al., 1998), Veber (GSK) (Daniel F. Veber et al., 2002), Egan (Pharmacia) (William J. Egan et al., 2000), and Muegge (Bayer) (Ingo Muegge et al., 2001).

Swissadme a fee web gateway for drug-likeness prediction unswervingly exhibited physiochemical properties in which molecular weight, number of hydrogen atoms, rotatable bonds, TPSA (topological polar surface area), molar refractivity were mentioned, then lipophilicity, water-solubility, pharmacokinetics, drug-likeness, and medicinal chemistry were also listed here and these all parameters can be evaluated by inserting the Canonical smiles of respective compound (Daina et al., 2017).

Shawky in 2019 reported drug-like properties of 6844 constituents by ADME filtration and 53 constituents were left behind after filtration linked by above 300 edges to 36 targets by network pharmacology approach and using Cytoscape plugins. (Shawky, 2019).

2.10 Drug-Target Relationship

(Bhadra, 2020) reported the in-silico assessment of Ajwain leaves on aster yellows disease and by receptor confinement method. They discover binding sites on targets proteins where phytochemical components of T.ammi can bind. They found targets of phytoconstituents of T.ammi from protein data bank (PDB).

One study reported different therapeutic targets for RA from different databases such as Therapeutic Target Database (TTD), Drugbank and Potential Drug Target Database (PDTD). 96 targets were retrieved from all these databases and then performed PANTHER analysis based on evolutionary relationships and functional examination of current RA potential targets in humans was accomplished using four approaches: molecular function, cellular compartments, biological process, and metabolic pathway engrossment (Di, 2011).

Analysis of previous studies about RA, *Trachyspermum Ammi* essential oil druglike bioactive compounds has helped in gaining insight into the extent to which these topics have to be researched and the gaps that needed to be filled in future research

METHODOLOGY

Biochemical Profiling of Trachyspermum Ammi

3.1 Collection of *Trachyspermum Ammi* (Ajwain) seeds

Ajwain seeds were purchased from the local market of Rawalpindi and verified from National Agricultural Research Center (NARC) Islamabad, Pakistan as *Trachyspermum Ammi* with accession number 38452.

3.2 Extract Preparation

3.2.1 Maceration

Maceration of Trachyspermum ammi plant was done according to (Soltani Howyzeh et al., 2018) and (Hammash et al., 1970) with slight modifications. Plant material was ground in a grinder to make a fine powder and extract was prepared in a 1:10 ratio in organic methanol and an aqueous solvent. 50 g of plant fine powder was taken and add 500ml of methanol and water in a separate reagent bottle which yields two types of extract, one is organic and the other is aqueous. Both the extracts were shaken three times a day and kept in dark for approximately 12 days to avoid adulteration of extract because they are light sensitive.

3.2.2 Filtration

After a maceration of 10-12 days, both the extracts were purified by the use of Whatman filter paper of pore size a 0.45 µm in a volumetric flask. During filtration, there was a need to avoid temperature and light exposure because of their light-sensitive nature. In this phase, the extract was in crude form and stored at 4^{o}C.

3.2.3 Drying

A half portion of both methanolic and aqueous crude extracts was allowed to air dry in an autoclaved Petri plate so that solvent evaporates and left behind a purified extract and kept in 4^{o}C until further used in experimentation. After drying percent yield was estimated by the subsequent formula (Gahlot et al., 2018).

$$\text{Percentage yield (\%)} = \frac{\text{Weight of dry extract}}{\text{Weight of Extract powder}} \times 100$$

3.3 Phytochemical Screening

Phytochemical analysis of both aqueous and methanolic extracts was occurred in two phases, qualitative and quantitative.

3.3.1 Qualitative Phytochemical screening

The qualitative phytochemical analysis (Hossein & Sakha, 2015) was addressed in table 3.1.

Table 3.1: Phytochemical analysis of T.ammi extracts

Sr.no	Test name	Procedure	Observations
1	**Phenol test**	1ml of 1% $FeCl_3$ solution and 1 ml of extract	Bluish Black colour
2	**Flavonoid Test**	1ml of 10% of lead acetate solution and 1 ml of extract	Yellow precipitates
3	**Alkaloid Test**	Hager's reagent is made by mixing 1g of picric acid in 100ml of water. 2 ml extract and add few drops of Hager's reagent.	Yellow precipitates
4	**Steroid test**	2ml extract and few drops of sulphuric acid and chloroform	Reddish-brown color
5	**Coumarins**	1ml of extract and 1.5ml NaOH (10%)	Yellow ppt
6	**Anthrocynin**	2ml extract and 2ml of 2N HCL and add 1ml of NH_3	Color change from pinkish red to bluish violet.
7	**Leucoanthrocynins**	1ml extract and 1ml isoamyl alcohol.	Red-orange layer
8	**Cardiac Glycosides**	2ml of acetic acid along with few drops of $FeCl_3$ add 1ml of concentrated sulphuric acid and	Violet to brown coloration

		add 2ml of extract	
9	**Diterpenes**	2ml extract and 2ml water along with few drops of copper sulfate solution	Emerald, green coloration
10	**Triterpenes**	1ml of extract in a glass tube and few drops of concentrated sulphuric acid. Shake slowly and allowed to stand	Yellow color in the lower layer.
11	**Tannins**	Braymer's testing in which 2ml of the extract was mixed with few drops of 5% $FeCl_3$	Transient greenish to black color formation
12	**Aminoacids**	Few drops of ninhydrin (1g of indane 1,2,3 trione hydrate was dissolved in 100ml n-butanol) and 1ml extract	Violet color formation

3.3.2 Quantitative Phytochemical screening

Quantitative screening of phytochemicals was illustrated by Total phenolic and flavonoid content in which Concentration of phenols and flavonoids was determined.

3.3.2.1 Total Phenolic Content

The total phenolic content in plant extracts was determined by using Folin-Ciocalteu colorimetric method based on oxidation-reduction reaction. The protocol was followed by (Genwali et al., 2013) and (Kamtekar et al., 2014) among slight modifications. Gallic acid was made in 1:1 in distilled water and aliquots in a series of dilution range from 100-900µg/ml was prepared. In a 15ml falcon tube 1ml of gallic acid in different concentrations was added and to that 10% Folin-Ciocalteu reagent (5ml) and 7% Na_2CO_3 (4ml) were combined to obtain a total volume of 10 ml. The mixture of blue color was properly shaken and heat for 30 minutes at 40^0C in incubator. At 760 nm the absorbance was measured against blank in Optima XP300 Spectrophotometer. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

Total phenolics content of the extracts was expressed as mg gallic acid equivalents (GAE) per 100 gram of sample in dry weight (mg/g) (Hammash et al., 1970), (Genwali et al., 2013).

3.3.2.2 Total Flavonoid Content

Total flavonoid content (TFC) was assessed by the aluminum chloride colorimetric assay. 1ml standard rutin solution in a concentration dependent manner was added into falcon tubes and distilled water (4ml) and 5% sodium nitrite (0.3ml) solution was added into each. After 5 minutes, 10 % aluminum chloride (0.3ml), 1 M sodium hydroxide (2ml) was added. At last, raise the volume up to 10ml with distilled water and shaken well. There was an appearance of orange yellow color. At 510 nm the absorbance was measured using Optima XP300 Spectrophotometer instrument. The blank was performed using distilled water. The calibration curve was plotted using standard Rutin. The data of total flavonoids of polyherbal formulation were expressed as mg of rutin equivalents/ 100 g of dry mass (Kamtekar et al., 2014), (Kalita et al., 2013).

3.4 Antioxidant Activity

3.4.1 DPPH Assay

The antioxidant activity of pure extracts of Trachyspermum ammi was evaluated through a DPPH assay by scavenging the free radicals. The protocol was followed by (Kumar Bajpai et al., 2014.) and (Chitme et al., 2016) with slight modifications. The whole experiment was done on 96 well plate and run-in triplicate with different series of dilutions of both extracts ranging from 10-80µg/ml concentrations. Ascorbic acid was taken as a control with the same dilution concentration. Ibuprofen was taken as a commercially available drug control. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was made in methanol 2:50. 2mg DPPH was added in 50ml methanol and kept on ice in dark. All the extract and ascorbic acid were diluted in 1:1 in methanol. The volume of 100µl for each concentration of extract, ascorbic acid, and Ibuprofen was prepared in 96 well plate and add 500µl of DPPH into each well. The reaction mixture was incubated for 1 hour at room temperature in dark. After incubation, the optical density (OD) was taken at 550nm by blank with the solvent that was used in extract and ascorbic acid preparation. Final readings were evaluated by using the formula:

$$\% \text{ Inhibition activity} = \left[\frac{\text{Abs of Control - Abs of Sample}}{\text{Abs of Control}} \right] \text{X } 100$$

3.4.2 Hydrogen Peroxide Radical Scavenging Assay

The capability of the extract to scavenge hydrogen peroxide (H_2O_2) was determined according to the method of (Bhatti et al., 2015). T. ammi methanolic and aqueous extracts (0.3ml), 50 mM phosphate buffer (0.9ml, pH 7.4) followed by the addition of 2mM H_2O_2 solution (1.8ml). The reaction mixture was vortexed and after 10 min of reaction time, its absorbance was measured at 330 nm on Optima XP300 spectrophotometer. Ascorbic acid was used as the positive control. The ability of the extracts to scavenge the H_2O_2 was calculated using the following equation (Kibiti & Afolayan, 2015).

$$H_2O_2 \text{ Inhibition } (\%) = \left[1 - \frac{H_2O_2 \text{ Conc of sample}}{H_2O_2 \text{ Conc of Blank}} \right] X\ 100$$

3.5 Protein Denaturation Inhibition assay

Protein denaturation assay was performed by following (Murugesan et al., 2020) with slight modifications. Both methanolic and aqueous extracts were made in 1:1 in distilled water and aliquots in a series of dilutions range from 100-500µg/ml. Add 1ml of extract and 0.1ml of 5% bovine albumin serum. The pH was adjusted to 7 and incubated at 37 °C for 20 min. Then the samples were heated at 57 °C for 30 min. After cooling the samples, 1.5 mL PBS (pH 6.3) was added to each tube and made up to 2.5 ml. Turbidity was determined in a spectrophotometer at 660 nm wavelength in Optima XP300 Spectrophotometer compared to control. The same steps are followed for the estimation of ascorbic acid and ibuprofen properties in protein denaturation. The percentage inhibition of protein denaturation was computed using the formula from (Alamgeer et al., 2015).

$$\text{Percentage Inhibition} = \left[\frac{\text{Abs of test Sample}}{\text{Abs of Control}} - 1 \right] X\ 100$$

3.6 Gas Chromatography-Mass Spectrometry of Ajwain

For GCMS 1g of dried powder of T. ammi seeds was dissolved in 1 liter of methanol and after 5 days filter it and allow to air dry. After that 1g of purified extract was taken and dissolved in the respective solvent and subjected to GC-MS in a QP-2020 SHIMADZU (Japan) system interfaced to SH-Rxi-5Sil mass spectrometer available in USP-CASE NUST. The system is equipped with a 1,4-bis (dimethyl siloxy) phenylene dimethyl poly-siloxane fused capillary

column with dimension o.25µm X 20m X 0.25mm. ionization energy was set at 70eV before initiating the analysis and the rest of the conditions were set according to the plant properties. For GC-MS detection of T.ammi, the oven temperature was maintained at 100^{o}C the flow rate of gas was set at 1ml/min. Structures of components detected in GC-MS analysis were identified by PubChem online server.

"In-Vivo analysis of anti-inflammatory and anti-arthritic potential of Trachyspermum Ammi"

3.7 Ethical Approval of mice model

All of the experiments on the mice model were conducted after taking the approval of the Institutional Review Board (IRB), performa number 32-IRB-ASAB-2016 from Atta-ur-Rehman School of Applied Biosciences (ASAB), and all of the experimental procedures were according to the guidelines described by the National Institute of Health (NIH).

3.8 LD 50 (Determination of Acute Lethal Toxicity)

According to the world health organization (WHO), the median lethal dose (LD_{50}) is the amount of extract or medicine or treatment affecting the death of 50% of treated animals. The protocol tracked for oral toxicity was conducted by (Chinedu et al., 2013) with slight modifications. For the oral LD50 determination 15 animals were used in five different groups (Four groups for toxicity testing of extract and one group as a normal control giving normal water) for three different phases shown in figure 3.1

Phase 1

In one study design it was reported that the initial dose started from 5, 50, 500, or 2000mg/kg of the mouse weight being chosen for the assessment of acute oral toxicity of the material being examined (Fu et al., 2014) so, in phase 1 mouse models were treated initially with 50, 150, 200 and 400mg/kg of T.ammi purified extract and check mortality within 24 hours.

Phase II

In the second phase treatment total 9 mice were taken for dose of 1000, 1500, 2000mg/kg of mouse weight were administered, and mortality rate was observed.

Phase III

In third phase mouse are treated with 3000, 4000 and 5000mg/kg of mouse were administered, and mortality was observed. After every phase mouse models were kept on simple water for approximately 24-30 hours to elute out previous remains of dose in the mice so that they are prepared for the next dose. Moreover, if the major test did not show up any transience at 5000 mg/kg, an assenting test should also be conducted. This can be done by processing 5000 mg/kg dose to animals. Examination should be done for 1, 4 and 24 hours after administration. The recording of no death should be an affirmation of the test result. Lethal Dose 50 (LD50) was then calculated by using the following formulae

$$LD50 = \frac{(M_O + M_1)}{2}$$

Where D_0 is the maximum dose where no death occurs while D_{100} is the minimum dose where mice died.

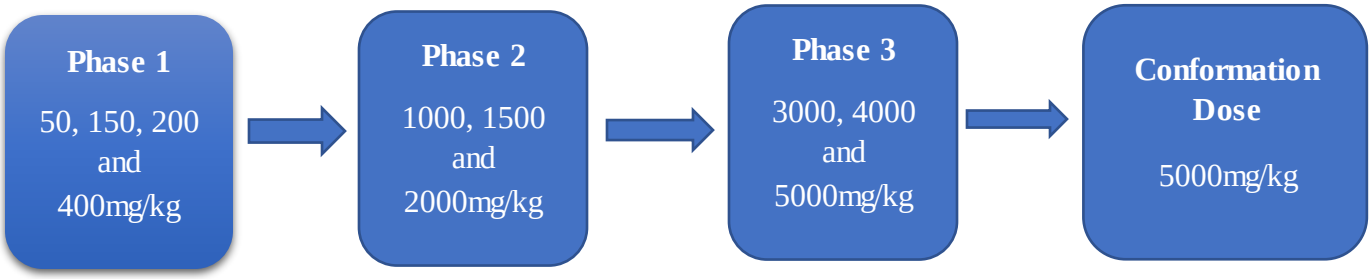


Figure 3.1: Schematic view of LD-50 of mice model.

3.9 Animal model Construction

The criteria of including animal model in a present study are listed in table 3.2.

Table 3.2: Animal inclusion criteria

Model	**Balb/c Mice**
Gender	Female
Weight	25-35 grams
Age	3-4 weeks
Sample Size	Total 24 mice (6 per group)

.

3.9.1 Animal Grouping and Acclimatization

24 female Balb/c mice of age 3-4 weeks were obtained from ASAB animal house, divided into four groups and each group contain six mice explained in table 3.3 and were housed at laboratory animal house ASAB. They were given and observed an acclimatization period of approximately 10 days before model construction to check whether any of the mouse model got tumors or diseases before aminal model construction.

Table 3.3: Grouping of an animal model into the experimental group

Cages	Animal groups for experimentation	No.of mice
1	Healthy Mouse model	6 mice
2	Arthritic diseases Model	6 mice
3	*Trachyspermum ammi* treated model	6 mice
4	Ibuprofen treated model	6 mice

3.9.2 Model establishment and arthritis induction

Collagen-induced arthritis mice model was established by injecting mice with type 2 collagen and Freund adjuvant. Four-degree arthritis was induced according to a reported protocol (Korani & Jamshidi, 2020) (Qamar et al., 2020).

1. Type 2 collagen was dissolved in 0.1M acetic acid in a 2:1 ratio and the mixture was kept overnight at 4^{o}C. 0.1 M acetic acid was made in 0.89% Hartman's Solution.
2. Type-II collagen was mixed with Complete Freund's adjuvant of concentration 2mg/ml at the ratio of 1:1 followed by vortex for 2–3 minutes to form a Completely homogenous and an immunization mixture.
3. Then BSA (1 mg/mL) dissolved in Hartmann solution (0.89%) was added to the immunization mixture at a ratio of 2:1.
4. The prepared immunization mixture of 0.2 mL or 200µl was injected into the mouse hind paw for localized disease activation (Cambré et al., 2018) on day 0, 7, and 14. On day 21 and 28 the booster doses of Complete Freund's adjuvant were administered in the same hind paw of mice. A schematic view of mouse model construction was drawn in Figure 3.2.
5. The evaluation of arthritic brutality was done by arthritic clinical scores from 0–4 mentioned in table 3.4. Grade 4 shows that arthritis was fully induced.

Table 3.4: Grading criteria of Arthritis induction

Sr. no	Condition	Grading score
1	Pivotal redness with no joint swelling	0
2	Slightly swollen finger joints	1
3	Swollen joints below the ankle joint	2
4	Inflammation on the whole paw, angiogenesis	3
5	Joint deformity, rheumatic nodules	4

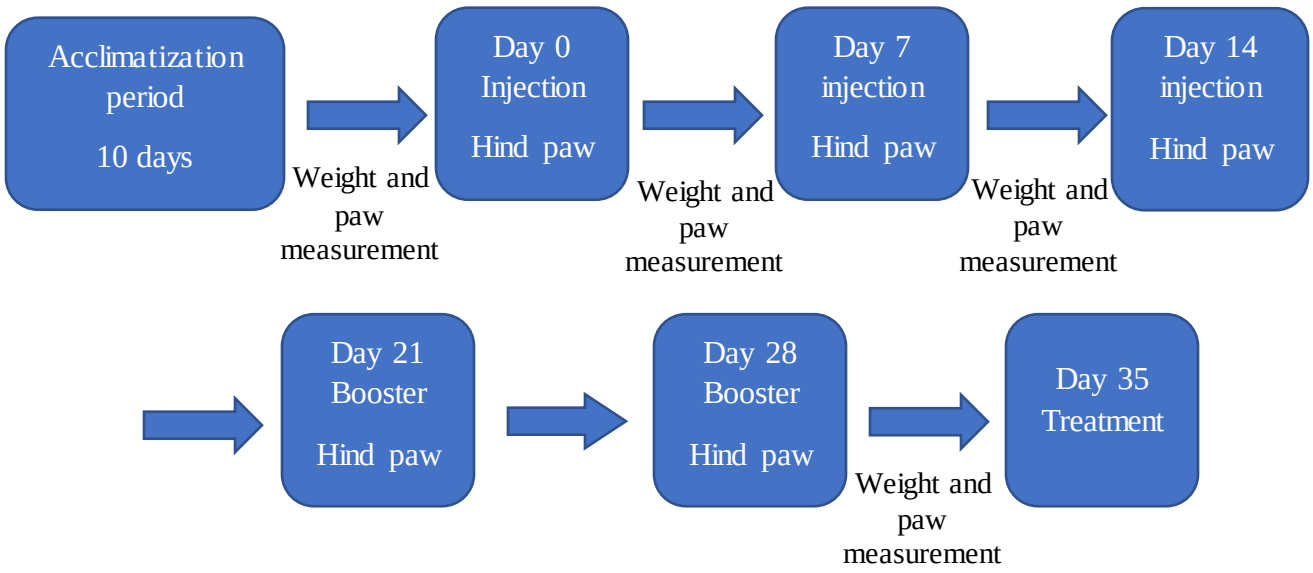


Figure 3.2: Schematic flow sheet of mouse model construction

3.9.3 Measurement of mice weight and paw

Weight and paw measurement of mice model was done once a week for consecutive 6 weeks of mouse model construction and treatment. Weight was measured by weighing balance and paw volume was taken with the aid of a vernier caliper. These measurements were also taken during 14 days of treatment spans and Shortly before the sacrifice of animals in the animal house ASAB.

3.9.4 Administration of Standard Drugs and Extract to the Mice model

The mouse model was given the treatment dose of 5000mg/kg which seems the highest dose of Acute oral toxicity (Chinedu et al., 2013). This dose was given for 14 days of the treatment period.

3.10 Catalase Assay

Catalase is one of the most important antioxidant enzymes that protect the cell against reactive oxygen species, generated as a result of oxidative stress. The major role of this enzyme is to catalyze the formation of water and oxygen from hydrogen peroxide (H2O2). The protocol is followed by (Dassprakash et al., 2012) and (Sinha, 1972) with slight modifications. In catalase assay, catalase present within the tissue sample degrades the H2O2 to generate water and oxygen molecules. Following steps were performed for catalase assay

Qualitative Assay

In this procedure, the solid liver homogenate was administered with 2M H2O2 and observe the formation of Oxygen bubbles from the degradation of H2O2 by catalase.

Quantitate Assay

Steps for quantitative tests of catalase assay are as follows:

1. 0.5g of liver tissue was crushed in 2.5ml of 0.01M cold PBS solution on dry ice because catalase is highly sensitive and works optimally on 37^{o}C and loss its activity if the temperature exceeds 37^{o}C.
2. Liver tissue homogenate was centrifuged at 7500rpm for 12 minutes at 4^{o}C.
3. Prepare 1.5ml solution mixture in which 1ml of 0.01M PBS (pH 7), 0.4ml of 2M H2O2, and 0.1 ml of liver tissue homogenate.
4. Incubate at room temperature for 1 minute.
5. Add 2ml of dichromate acetic acid reagent (5% potassium dichromate sol into glacial acetic acid) at 1:3 volume.
6. Incubate for 10 minutes at 37^{o}C.
7. Measure absorbance at 620nm.

Results of Absorbance were analyzed by using formula and The activity of catalase in tissue homogenate was expressed as μ moles of H_2O_2 utilized/min/mg protein.

$$\text{Formula} = \frac{\text{Abs of standard} - \text{Abs of Sample}}{\text{Abs of Standard}} \times \frac{1}{\text{Gram of tissue used per test}}$$

3.11 Spleen Indexing

Spleen indexing was dome by the protocol of (Li et al., 2017). Wight of mice was measured before sacrifice and after sacrifice measure weight of spleen. The ratio of spleen weight to the total body weight of the mouse is known as spleen index and was calculated by using the formula:

$$\text{Spleen index} = \frac{\text{Wight of spleen (mg)}}{\text{Weight of body (mg)}} \times 100$$

3.12 Clinical Chemistry

The liver and kidney are the main organs that effect by toxicity and any type of treatment by damaging its physical and functional integrity which can be measured by Liver Function tests (LFTs) and Renal Functional tests (RFTs). The functional deformity can assessed by levels of liver function-correlated enzymes such as alanine transaminase (ALT), alkaline phosphatase (ALP) (Shakibaie et al., 2012), and serum bilirubin (Dash et al., 2007) (Sher & Hung, 2013). Similarly, for kidney dysfunction, RFTs including serum urea and creatinine were performed (Kareshk, 2018). These tests were performed for evaluating toxicity in LD-50 of mice model and arthritic mouse model after model construction and treatment. Blood was collected in yellow vacutainers and send to ASAB diagnostic Lab NUST Islamabad for estimation of LFTs and RFTs.

3.13 Histopathology

After arthritic induction, mice were sacrificed, and paws were collected from mice for histopathology. Histopathology was done in the National Institute of Health (NIH) Islamabad to see tissue morphology and to study the appearance of RA disease in ankle joints. Paws were taken for histopathological analysis so that changes in paw can be studied after arthritic induction and before and after administration of two extracts. After sacrifice paw was collected in 10%

formalin at -80 until further processing. Paws were immersed in 4% nitric acid overnight for decalcification of bones involved in joints for good sectioning. The next step is a fixation for these paws were then immersed in 70%, 80%, and 100% isopropanol for 2 hours. After this, they were immersed in 50:50 and then 30:70 ratio of isopropanol: xylene each for two hours. Xylene was used to wash out alcohol and allow infiltration with paraffin wax. The next step is embedding, in which specimens were infiltrated with an embedding agent (paraffin wax). Tissues are surrounded by a large block of molten paraffin wax and it is named a block. Block as solidifies provide support for thin and easy sectioning. Blocks were chilled in the refrigerator for 10 minutes before sectioning. Tissue specimen blocks were then sliced by microtome into thin sections in form of a ribbon and placed gently in a warm water bath. Here sample was allowed to float on water. Then thin tissue ribbon was scooped up onto a slide placed under the water level. Slides had adhesive on them which helped in easy capturing of tissue specimens on a slide. This slide is placed in a hot plate for the removal of excess paraffin wax from the slide surface leaving behind the exposed tissue. The tissue remains colorless when they were unstained. The final step was staining, in which slides were kept all together in xylene 1 chamber for 10 minutes and then transfer to the next xylene 2 chamber and kept there for 10 minutes. Slides were again transferred to the next chambers containing absolute alcohol 1 and absolute alcohol 2 and kept there for 5 minutes each. After this, slides were again transferred to a chamber containing 70% ethanol and 50 % ethanol and kept there for 2 minutes and 1 minute respectively. Then slides were washed under running tap water. The next step involved dying with hematoxylin, their slides were kept in this chamber for 3 minutes and they were then washed under running water. Again slides were kept in an eosin dye chamber for 20 minutes. This staining helped in providing contrast to tissue sections and making tissue structure more visible and easier to evaluate. They were again washed with water and dehydrated with ethanol. In last slides were mounted over with coverslips using optical aid oil which helps in the protection of tissue specimens and easy visibility.

Microscopy was performed at Khan Research Laboratories (KRL) Islamabad at 20X, 40X, and 100X magnification power.

"In-silico evaluation of Trachyspermum Ammi"

3.14 Data Preparation

GCMS data of T.ammi contains 933 bioactive compounds and Drug like compounds are shortlisted or identified by drug likeliness and ADME screening.

3.14.1 Identification of Druglike compounds

Recognition of compound was done by **PubChem** (https://pubchem.ncbi.nlm.nih.gov/) in which compound ID, molecular formula, IUPAC name, InChlKey, Physiochemical properties, canonical smiles, 2D and 3D structures, and much other information is given (Kim et al., 2016). To check druglike prediction, compounds were analyzed by freely available web bioinformatic tool Swissadme (http://www.swissadme.ch/) and for calculation of molecular properties and bioactivity score molinspiration (https://www.molinspiration.com/cgi-bin/properties) is used. By entering canonical smiles in the software physiochemical properties, Lipophilicity, water solubility pharmacokinetics, medicinal chemistry, and drug likeliness can be checked through Swissadme for evaluation of druglike bioactive compounds. Compounds were filtered by following the lipinski rule of five (ROF) parameters proposed by (Lipinski et al., 1997), ghoose rule (Arup K. Ghose et al., 1998), Veber rule (Daniel F. Veber et al., 2002), Egan rule (William J. Egan et al., 2000), Muegge rule (Ingo Muegge et al., 2001). Details of these rules are listed in Table 3.5. Compounds violating any one or more rules were eliminated and the rest were again evaluated through ADMET screening. For ADMET screening (absorption, distribution, metabolism, excretion, Toxicity) and quantitative structure-activity relationship (QSAR) modeling analysis, free web silico tool **admetSAR** (http://lmmd.ecust.edu.cn/admetsar2) is used in which canonical smiles of compounds were entered and they were shortlisted by validating certain parameters like human intestinal absorption, Caco 2 permeability, Blood-brain barrier, human oral availability, p-glycoprotein substrate, ames mutagenesis, human either a-go-go inhibition, hepatotoxicity, acute oral toxicity, and binary and trinary carcinogenicity.

Table 3.5: Druglike prediction parameters of Lipinski, ghose, veber, egan, and muegge rule.

	Parameters	Range
Lipinski Rule	Molecular weight	≤ 500
	MLOGP	≤ 4.5
	Hydrogen Bond Donors (NH/OH)	≤ 5
	Hydrogen Bond acceptors (N/O)	≤ 10
Ghose Rule	Molecular weight	160 to 480
	WLOGP	≤ 5.6
	Molar Refractivity	≤ 130
	Heavy atoms	≤ 70
Veber Rule	Total polar surface area	≤ 140
	Number of rotatable bonds	≤ 10
Egan Rule	WLOGP	≤ 5.88
	Total polar surface area	≤ 131.6
Muegge Rule	Molecular weight	200 to 600
	XLOGP	-2 to 5
	Hydrogen Bond Donors (NH/OH)	≤ 5
	Hydrogen Bond acceptors (N/O)	≤ 10
	Total polar surface area	≤ 150
	Number of rotatable bonds	≤ 15
	Number of rings	≤ 7
	Number of heteroatoms	> 1

3.14.2 BOILED-EGG plot

Shortlisted compounds were again filtered for the precision of data so, blood-brain barrier (BBB) and Human Intestinal Absorption (HIA) were calculated by Brain Or IntestinaL EstimateD (BOILED- Egg) permeation method which was developed through SwissADME software. BOILED EGG plot examines the polarity [Topological Polar Surface Area (TPSA)] and lipophilicity (WLOGP) of a compound and predicts its pharmacokinetics concerning BBB and HIA (Daina & Zoete, 2016). TPSA is explained as the aggregate of the surface of polar atoms present in the compound chiefly nitrogen, oxygen, and attached hydrogen. Compounds

having TPSAof more than 140 angstroms squared are poor in absorbance in the GI tract while TPSA of lesser than 80 is needed to cross the BBB (Pajouhesh & Lenz, 2005). Lipophilicity (abbreviated as logP) is defined as a molecular factor that codes for both electrostatic and hydrophobic intramolecular and intermolecular forces of interactions. Compounds that are highly hydrophobic in nature gets partitioned within the lipid portion of the cell membrane, therefore, can't penetrate through the BBB, therefore, LogP value in the range of 0.5 to 5.9 can cross the BBB but the compounds lies in the range of 1.5 and 2.7 are the best ones to cross BBB (Flynn, 1980). If the point of a certain compound comes in the white portion of BOILED EGG then it has a high probability to get absorbed within the GI tract. Points located in the yellow portion (depicted as the yolk of the egg) have a high probability of crossing the BBB and get contact with CNS. There is a grey area in the graph as well; compounds that are predicted to be unabsorbed through the GI tract or BBB are pointed in this grey area.

3.14.3 MOLSOFT

At last, compounds were shortlisted based on the Blood-brain barrier by online tool molsoft drug-likeness prediction (http://molsoft.com/mprop/).

3.14.4 Therapeutic Targets of Rheumatoid Arthritis

Therapeutic targets for the treatment of Rheumatoid Arthritis can be retrieved from four sources and databases: **1)** Literature from following sources (Di, 2011), (Klein & Gay, 2015), (Koenders & Van Den Berg, 2015), (Cheung & McInnes, 2017), (Sanchez-Lopez et al., 2019), (Okamoto et al., 2008), (van den Berg & Miossec, 2009), (Burmester et al., 2014), (Karami et al., 2019), (Venuturupalli, 2017), (Tristano, 2009). **2)** Therapeutic Target Database (TTD) (http://db.idrblab.net/ttd/) **3)** Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/).

3.14.5 Prediction of targets of T.ammi Compounds

Therapeutic targets of lead compounds were retrieved from Swiss target prediction (STP) and Comparative Toxicogenomics Database (CTD) and Target Hunter (https://www.cbligand.org/TargetHunter/search_target.php). After assembling the data all targets names was verified by uniport (https://www.uniprot.org/) to explore for target genes given name for human species.

3.15 PPI enriched RA network and Clustering

The protein-protein interaction network of Rheumatoid arthritis targets was constructed by **Bisogenet 3.0.0 plugin** which promptly retrieves 6 accessible genetic databases (Martin et al., 2010). It was being made for analysis Parameters of bisogenet were set as follows:

1. Bisogenet plugin was installed in the apps of Cytoscape.
2. Put the "Organism" preference to "Homo Sapiens" in the identifiers set.
3. Programmed "Biorelation types in the Data" settings to MINT, HPRD, BIOGRID, DIP, INTACT algorithms.
4. In "Method" settings, to establish a network is aim to "input nodes only"
5. Output settings were maintained to embody nodes of network in positions of "Genes".

3.16 Networks Formation

All the networks were constructed online through STRING interaction networks (https://string-db.org/) and send to network visualization software Cytoscape version 3.8.2 (Hu et al., 2019) download from https://cytoscape.org/ for further analysis.

3.16.1 Network Establishment

Networks can be extablished by succeeding the following strategy:

1. Network of RA disease.
2. T.ammi bioactive compounds target network.
3. Intersection Merging of RA disease and the compound target network.
4. Disease-targets-mechanism pathway network.

On the STRING website, all the retrieved targets of Rheumatoid arthritis and bioactive compounds are pasted in the protein sequence search bar, and select multiple protein options and then select *Homo Sapiens* to construct a network. After the formation of networks in STRING, they were sent to Cytoscape individually and then merged each of the bioactive compound target networks to RA disease target network by Cytoscape tool merged networks in the intersection so that common targets in between were addressed. All the networks were analyzed by the Network

analyzer plugin. RA disease target - compound target – pathway network was made after enrichment. Merged targets functions were retrieved from Uniprot.

3.17 Enrichment and Pathway Analysis

Enrichment analysis of all the targets in merged networks was done by Enrichr (https://maayanlab.cloud/Enrichr/) to bring out Gene Ontology (GO) enrichment analysis in biological processes, molecular functions, and cellular compartments and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways prediction.

3.18 Docking Analysis

3.18.1 Establishment of Compound/Ligand library

Once compounds were shortlisted from the ADMET screening their three-dimensional structures were downloaded from the PubChem in SDF format (Kim et al., 2016). These compounds were made as ligands after energy minimization in the **PyRx** virtual screening tool (Dallakyan & Olson, 2015).

3.18.2 Preparation of target proteins

Enriched target proteins taken from GO enrichment were used for docking analysis for their respective compound/ligand. 3D structures of all targets were available on Protein Data Bank (PDB) (https://www.rcsb.org/) from where PDB files were downloaded (Berman et al., 2003) and opened in PyRx and made as macromolecule after energy minimization.

3.18.3 Molecular docking

All the enriched merged target proteins are docked with their respective bioactive shortlisted compounds or ligand by the PyRx virtual screening tool (Dallakyan & Olson, 2015).

3.18.4 Molecular visualization

Docked ligand with protein is visualized through **BIOVIA Discovery Studio Visualizer 2021** downloaded from (https://discover.3ds.com/discovery-studio-visualizer-download) (Sahu et al., 2019).

3.19 Statistical analysis

All the data concerning the anti-arthritic study are expressed as mean ± SD. Statistical analysis was presented by student t test, simple linear regression, one-way ANOVA followed by Tukkey's, and two-way ANOVA. followed by Bonferroni multiple comparison Post hoc tests using the "GraphPad-Prism" statistic computer program. A difference in the mean values of $p > 0.05$ was measured as statistically significant.

RESULTS

"In-vitro analysis/ Biochemical Profiling"

4.1 Extract Preparation

Both methanolic and aqueous extracts were prepared in approximately 10-12 days and after filter cruse extract was obtained and after air drying, in petri plates, the purified extract was obtained. % Yield was calculated from 500ml liquid extracts was addressed in table 4.1.

Crude Extract

Crude extract of methanolic extract was yellow and was clear whereas crude extract of aqueous extract was dark brown.

Purified/Dried Extract

Air-dried extract of methanolic extract got the appearance of brown color and became sticky because of evaporation of solvent whereas air-dried extract of aqueous extracts got the appearance of brown-black color and became dry due to evaporation of water.

Table 4.1: Yield of methanolic and aqueous extracts in grams and percentage

Sr. no	Solvent	Yield of Extracts	
		g	**%**
1	Methanol	13.25	26.5
2	Distilled Water	12.03	24.06

4.2 Phytochemical Screening

4.2.1 Qualitative Phytochemical screening

The qualitative phytochemical analysis of aqueous and methanolic extracts of the *Trachyspermum Ammi* plant was described in previous Chapter 3 and the results of both the extracts were summarized in table 4.2 and shown in figure 4.1-4.12 which shows results for both extracts along with its controls.

Table 4.2: Phytochemical analysis obtained from aqueous and methanolic extracts of *Trachyspermum ammi* plant. +++ sign shows strong present, ++ shows moderate present, + shows weak present whereas – shows absence

Test	Aqueous	Methanolic	Result Observation
Phenol test	+++	+++	Bluish black color
Flavonoid test	+++	+++	Yellow ppt
Alkaloid test	+	_	Yellow ppt
Steroid test	+++	_	Reddish brown color
Coumarins	++	+	Yellow ppt
Anthrocynin	_	_	Pinkish red to bluish violet coloration
Leucoanthrocynins	++++	_	Red Orange layer
Cardiac Gycosides	++++	+++	Violet to brown coloration
Diterpenes	++	+++	Appearance of emerald green color
Triterpenes	++	+	Yellow color in lower layer
Tannins	+++	+++	Transient greenish to black color formation
Aminoacids	_	_	Violet color formation

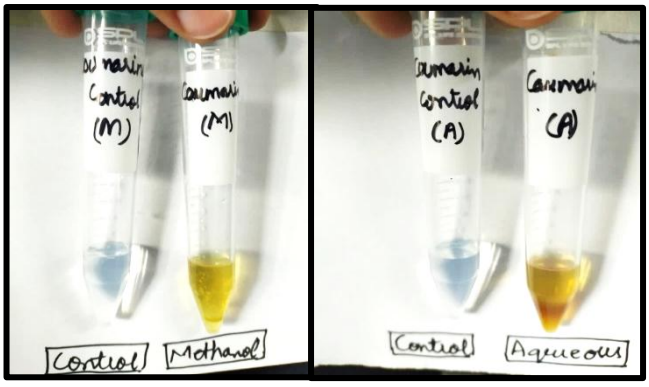


Figure 4.1: Positive coumarins test of aqueous and methanolic extracts of T.ammi

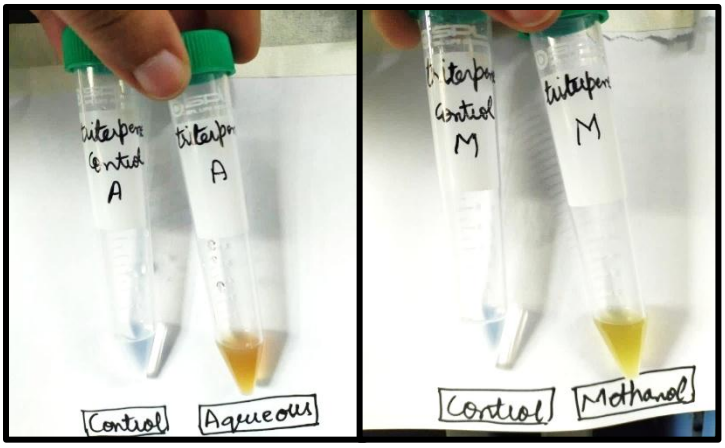


Figure 4.2: Positive triterpenes test of aqueous and methanolic extracts of T.ammi

Figure 4.3: Steroid test positive for aqueous and negative for methanolic extracts of T.ammi

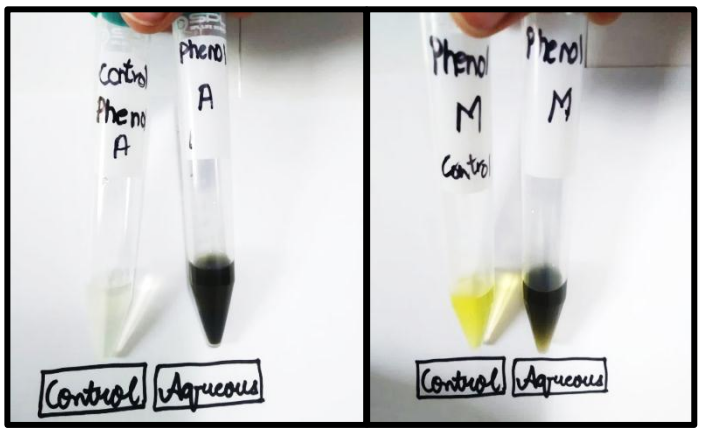


Figure 4.4: Positive Phenol test of aqueous and methanolic extracts of T.ammi

Figure 4.5: Negative Anthrocynin test of aqueous and methanolic extracts of T.ammi

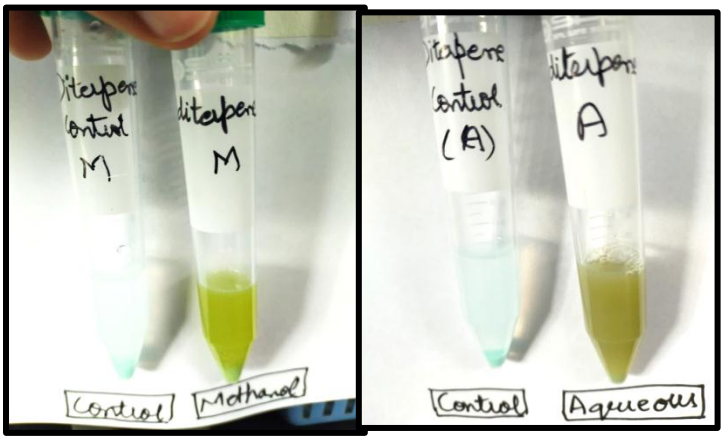


Figure 4.6: Positive diterpenes test of aqueous and methanolic extracts of T.ammi

Figure 4.7: Positive Flavonoid test of aqueous and methanolic extracts of T.ammi

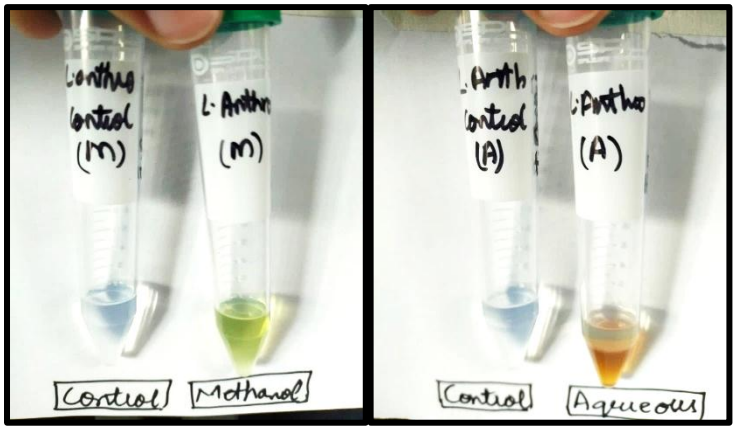


Figure 4.8: Leucoanthrocynins test positive for aqueous and negative methanolic extracts of T.ammi

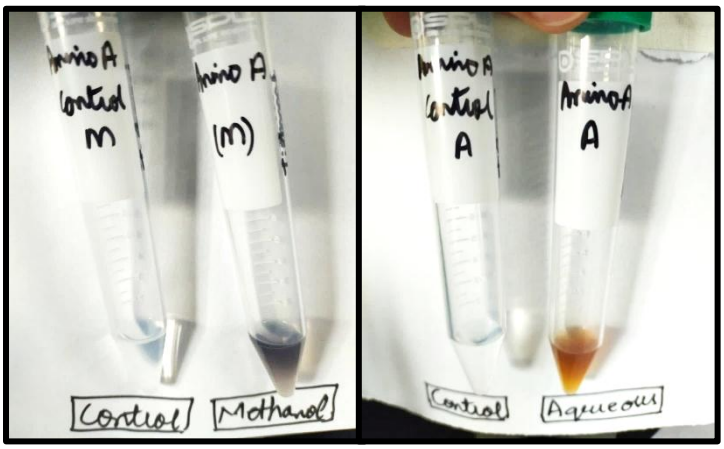


Figure 4.9: Negative ammino acid test of aqueous and methanolic extracts of T.ammi

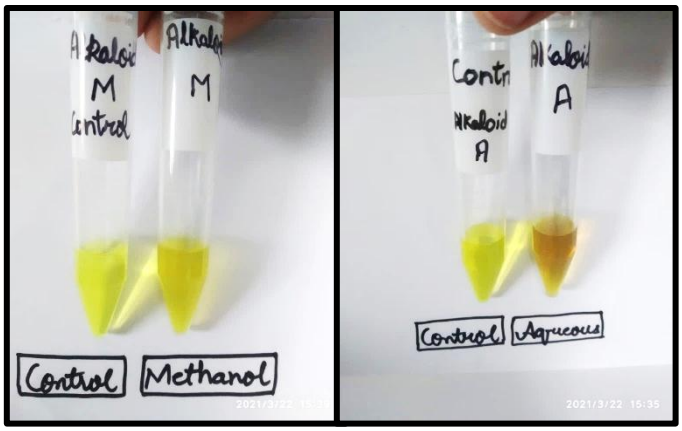


Figure 4.10: Alkaloid test positive for aqueous and negative for methanolic extracts of T.ammi

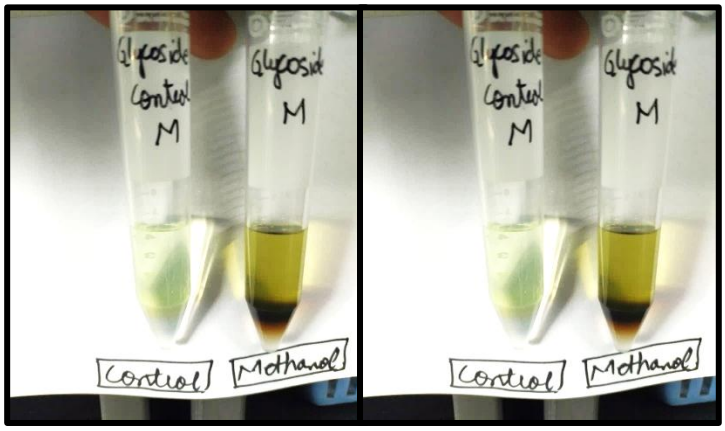


Figure 4.11: Positive cardiac glycosides test of aqueous and methanolic extracts of T.ammi

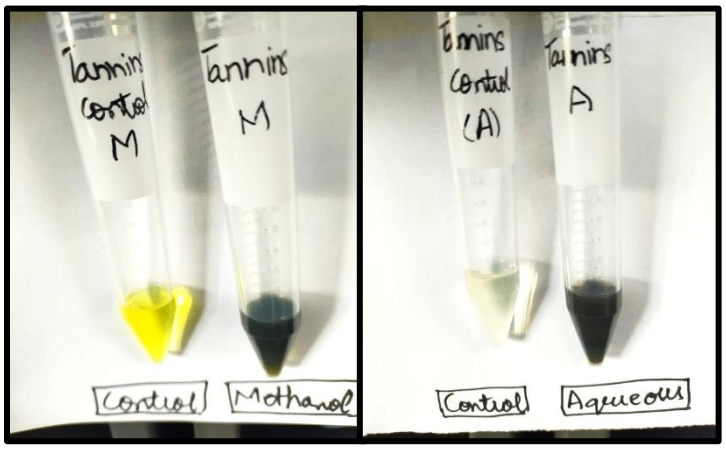


Figure 4.12: Positive tannins test of aqueous and methanolic extracts of T.ammi

4.2.2 Quantitative Phytochemical screening

4.2.2.1 Total phenolic Content

Total phenolic content (TPC) in different extracts was determined by Folin-Ciocalteu (F-C) method using gallic acid as the standard. The absorbance values obtained at different concentrations of gallic acid were used for the construction of the calibration curve figure 4.13. Total phenolic content of methanolic and aqueous extracts of *Trachyspermum ammi* was calculated from the regression equation of calibration curve and expressed as mg gallic acid equivalents (GE) per gram of sample in dry weight (mg/g). The results are presented in table 4.3 which declared that the TPC of methanolic extract was higher as compared to aqueous extracts of *Trachyspermum ammi.* Results were expressed as ± SD.

Table 4.3: Total phenolic content of methanolic and aqueous extracts of T.ammi.

Sr.no	Plant Extracts	TPC (mg/g) GAE
1	Methanolic extract	48.27±0.023
2	Aqueous Extract	15.4±0.064

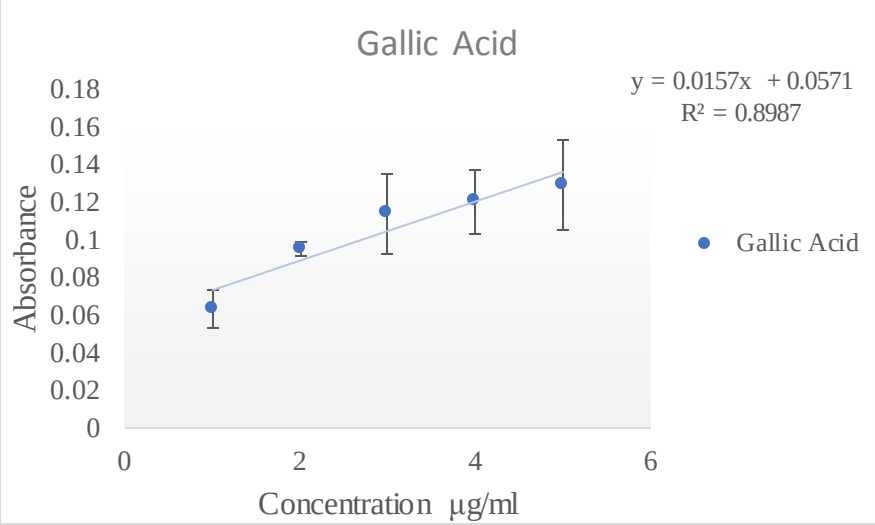


Figure 4.13 Calibration curve of standard Gallic acid.

4.2.2.2 Total Flavonoid Content

Total flavonoid content (TFC) in different extracts was determined by aluminum chloride colorimetric assay using Rutin as the standard. The calibration curve of rutin was obtained by plotting different absorbance from different concentrations. figure 4.14. Total flavonoid content of methanolic and aqueous extracts of *Trachyspermum ammi* was calculated from the regression equation of calibration curve and expressed as rutin equivalents (GE) per gram of sample in dry weight (mg/g). The results are presented in table 4.4 which declared that the TFC of methanolic extract was higher as compared to aqueous extracts of *Trachyspermum ammi*. Results were expressed as ± SD.

Table 4.4: Total Flavonoid content of methanolic and aqueous extracts of T.ammi.

Sr.no	Plant Extracts	TFC (mg/g) GAE
1	Methanolic extract	22.27±0.0156
2	Aqueous Extract	19.42±0.0566

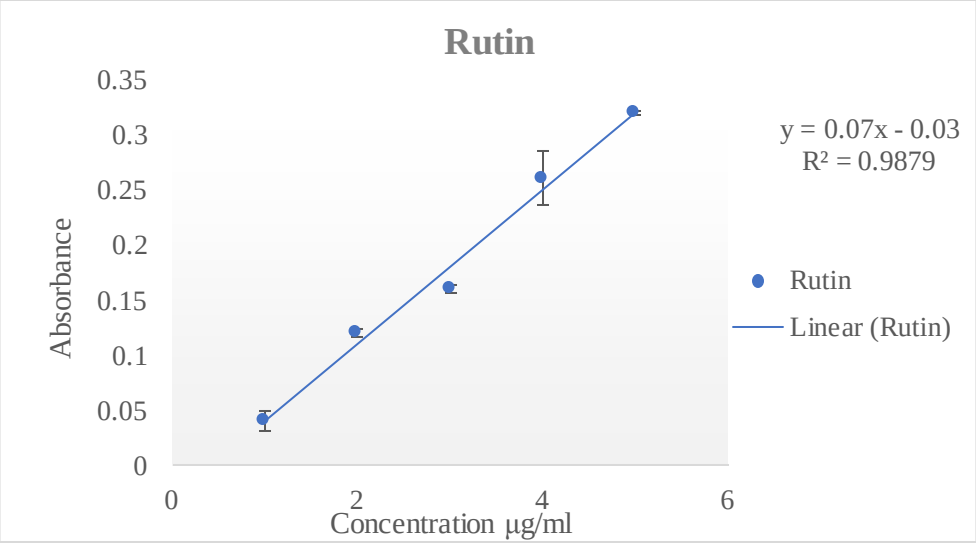


Figure 4.14: Calibration curve of standard Rutin.

4.3 Antioxidant Activity

4.3.1 DPPH Assay

DPPH assay was carried out to check antioxidant potential in both extracts. Results were assessed by finding % inhibition of the absorbance got from the 96 well plate reader of both extracts, ascorbic acid which acts as standard, and ibuprofen which is a commercialized drug. Results of all these categories were summarized in graphs figure 4.15 and 4.16. Graphs were obtained by applying linear regression. Results summarized that % inhibition of aqueous and methanolic extracts were greater as compared to standard ascorbic and originally commercialized drug ibuprofen which means T.ammi extracts had significantly highest antioxidant potential as compared to ascorbic acid and ibuprofen with P-value < 0.05. The antioxidant potential of T.ammi methanolic extract was slightly higher as compared to aqueous extract. Statistical analysis was listed in the table.4.5

Table 4.5: Statistical analysis of DPPH Assay

Sr. no	Sample	P value	R^2
1	T.ammi (M)	0.004	0.9003
2	T.ammi (A)	0.024	0.7575
3	Ascorbic acid	0.006	0.8858
4	Ibuprofen	<0.001	0.9898

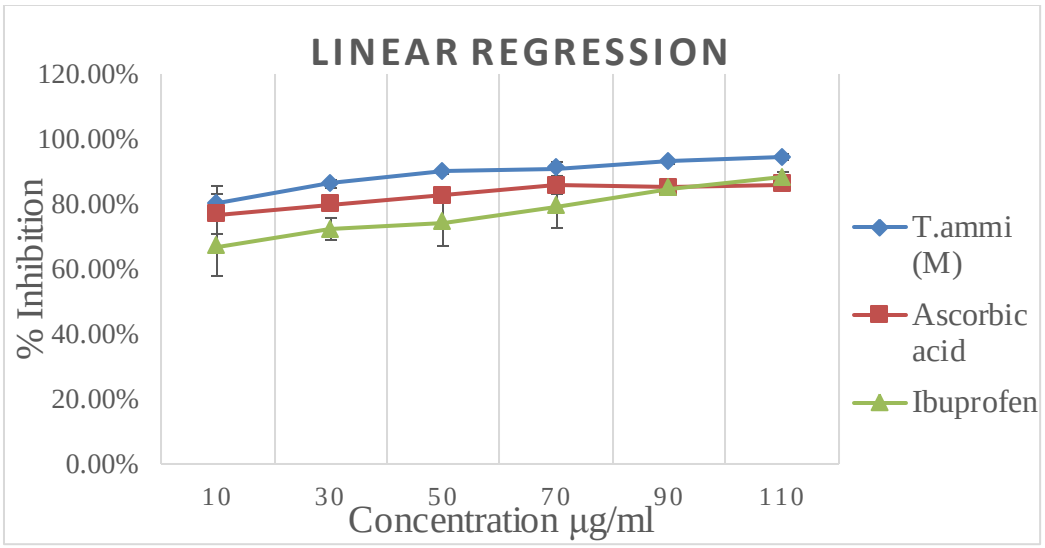


Figure 4.15: DPPH activity of methanolic extracts of *Trachyspermum ammi.*

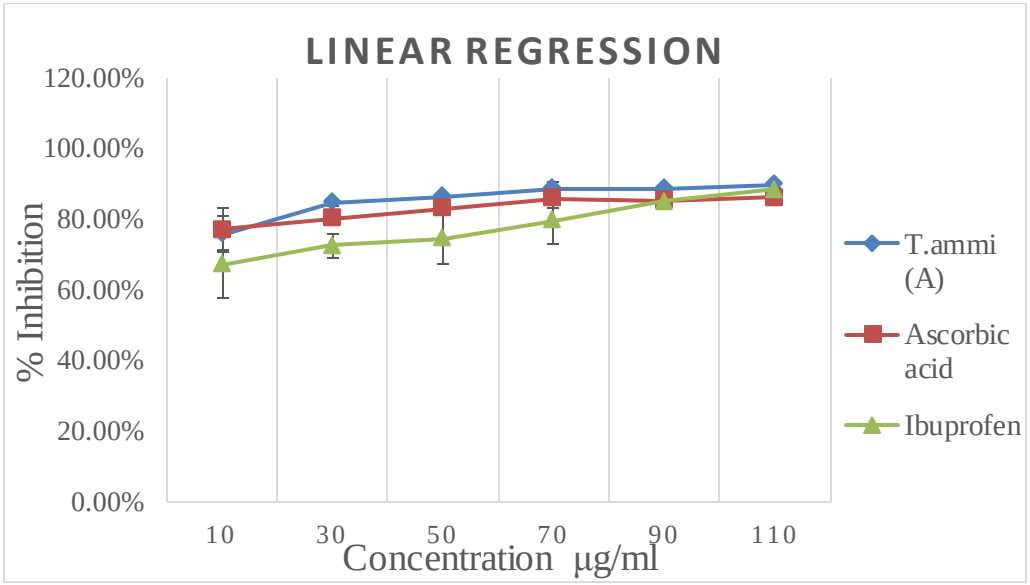


Figure 4.16: DPPH activity of Aqueous extracts of *Trachyspermum ammi.*

4.3.2 Hydrogen Peroxide Radical Scavenging Assay

Hydrogen peroxide scavenging activity of both the extracts, ascorbic acid and ibuprofen increasing with increasing concentration because extracts have antioxidant properties which were responsible for inhibiting reactive oxygen species (ROS) produce in the reaction mixture results in a decrease in absorption. Scavenging of H2O2 by extracts may be attributed to their phenolics, which can donate electrons to H2O2, thus neutralizing it to water. Although hydrogen peroxide

itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H2O2 is very important.

Thus, aqueous and methanolic extracts exhibit the highest scavenging activity as compared to standard ascorbic acid and commercialized drug ibuprofen figure 4.17 and 4.18 by decreasing H2O2 content. Results were analyzed by linear regression R^2 statistically significant at $p < 0.05$ listed in table 4.6.

Table 4.6 Statistical Analysis of Hydrogen Peroxide Radical Scavenging Assay.

Sr.no	Sample	P value	R^2
1	T.ammi (M)	< 0.001	0.9969
2	T.ammi (A)	< 0.001	0.9963
3	Ascorbic acid	0.003	0.9662
4	Ibuprofen	<0.001	0.9889

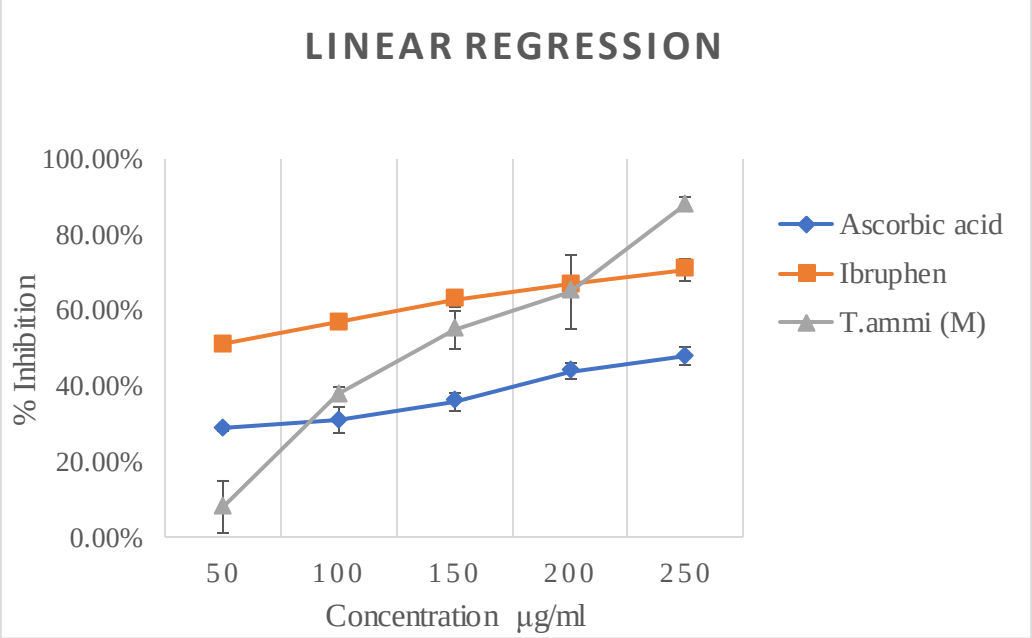


Figure 4.17: Hydrogen Peroxide Radical Scavenging Assay of aqueous extracts of T.ammi (A).

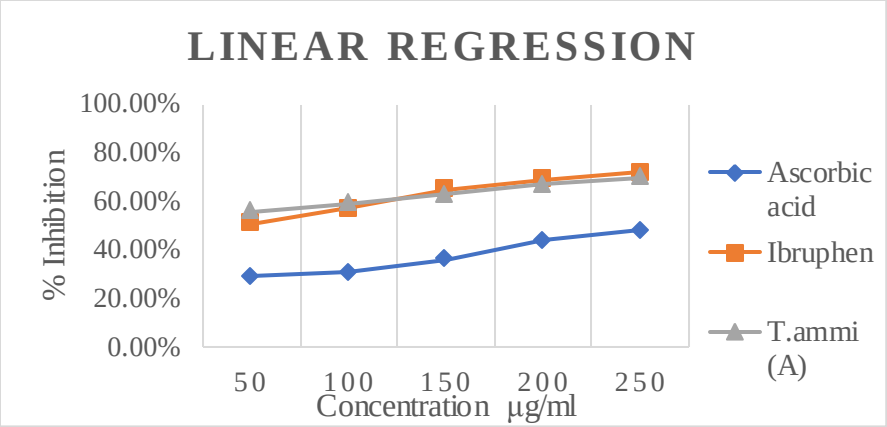


Figure 4.18: Hydrogen Peroxide Radical Scavenging Assay of methanolic extracts of T.ammi.

4.4 Protein denaturation inhibition assay

Protein denaturation has been identifying as a cause of inflammation. NSAIDs and DMARDS bind to plasma albumin preventing or inhibiting the thermal denaturation of albumin. Protein denaturation has been described as a pathological process that involves the loss of configuration, and as a result, loss of functionality. This reduces protein denaturation, and by extension the BSA protein denaturation assay, ideal for the determination of anti-inflammatory potential. It was observed by the assay that methanolic and aqueous extracts of the *Trachyspermum Ammi* showed maximum BSA inhibition potential as compared to standard ascorbic acid and commercially available drug ibuprofen which cause a reduction in inflammation. It can also be observed that methanolic extracts have higher inhibition potential as compared to aqueous extracts on increasing concentration. So, results reported that T.ammi methanolic extracts possess a higher protein denaturation inhibition potential Shows in figures 4.19 and 4.20. Results were analyzed by linear regression R^2 statistically significant at $p < 0.05$ listed in table 4.7.

Table 4.7: Statistical Analysis of Protein denaturation inhibition assay.

Sr.no	Sample	P value	R^2
1	T.ammi (M)	0.005	0.9499
2	T.ammi (A)	0.005	0.9504
3	Ascorbic acid	0.022	0.8663
4	Ibuprofen	0.001	0.9821

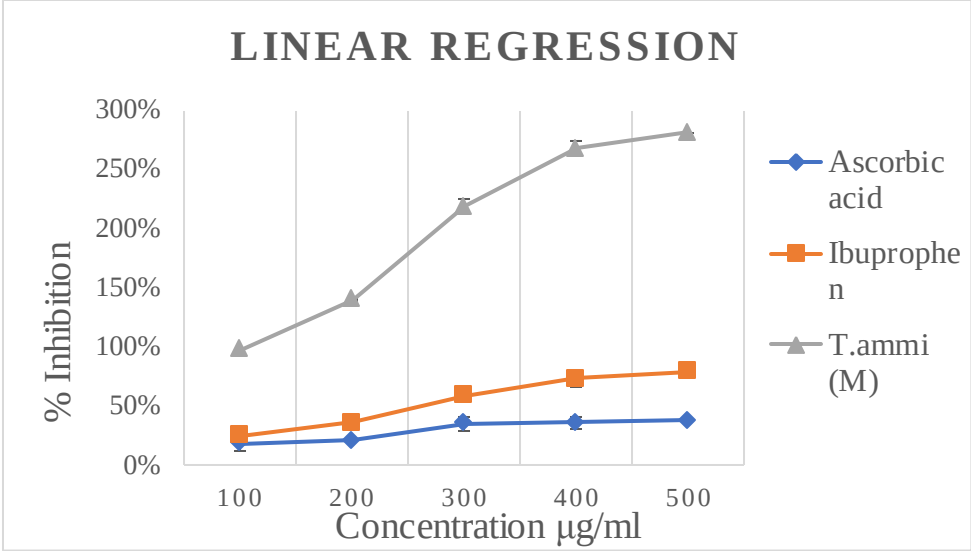


Figure 4.19: Bovine serum albumin denaturation inhibition activity of T.ammi methanolic extract.

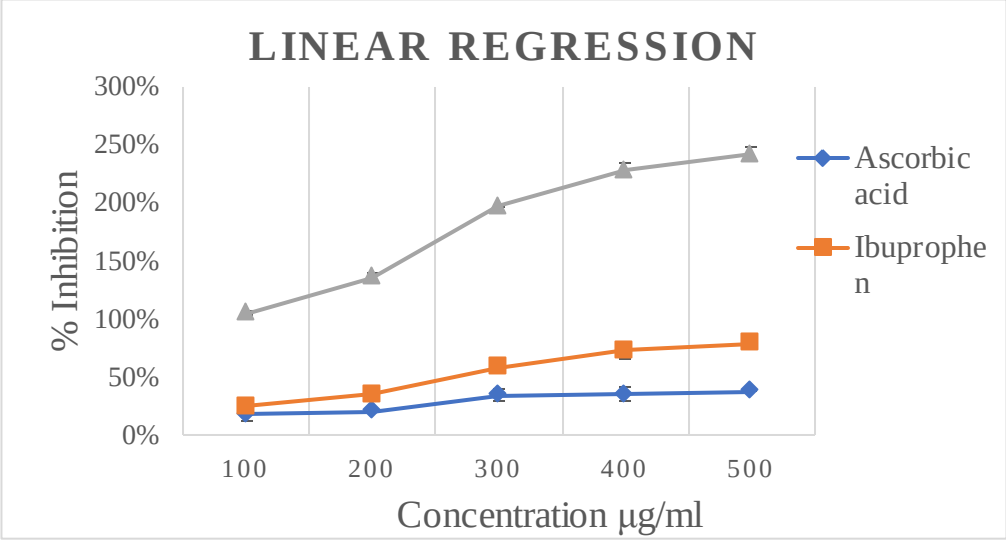


Figure 4.20: Bovine serum albumin denaturation inhibition activity of T.ammi Aqueous extract.

4.5 GC-MS Analysis

From the approval of the above plant profiling tests, it can be proved that T.ammi methanolic extract had the highest anti-inflammatory and antioxidant activity and have more

content of phenols and flavonoids. So, T.ammi methanolic extracts were used for further studies such as GC-MS and in vivo studies.

"In-Vivo Analysis"

4.6 LD 50 (Determination of Acute Lethal Toxicity)

Acute oral toxicity was carried on methanolic extract of *Trachyspermum ammi* because of its higher antioxidant and anti-inflammatory potential as compared to aqueous extracts, proved by in-vitro analysis of extracts. LD-50 observations of toxicity were described below:

- **Phase 1:** Mice were given doses of 5, 50, 500, or 2000mg/kg of the mouse weight and it was observed that all mice were alive showing no mortality against methanolic extracts of T.ammi.
- **Phase II:** Mice were given doses of 1000, 1500, 2000mg/kg of mouse weight and it was observed that all mice were alive showing no mortality against methanolic extracts of T. ammi.
- **Phase III:** Mice were given a dose of 3000, 4000, and 5000mg/kg of the mouse weight and it was observed that all mice were alive showing no mortality against methanolic extracts of T. ammi.

Then confirmation dose of 5000mg/kg of the mouse weight was administered but mice were still alive showing no mortality. These observations concluded that mice showed no toxicity against T. ammi extracts as reported by the Enegide method (table 4.8)

Table 4.8: Dose Toxicity Scale

Dose concentration (Enegide method)	Interpretation if shows signs of toxicity
1 or less mg/kg	Extremely Toxic
1-50 mg/kg	Highly Toxic
50-500 mg/kg	Moderately Toxic
500-5000 mg/kg	Slightly toxic

4.7 CIA Mouse Model Construction

4.7.1 Treatment with extracts

Mice with four-degree arthritis were chosen for treatment and divided into three groups of which one controls for the arthritis disease model, one group was treated with T.ammi methanolic extracts and one group was treated with commercialized drug ibuprofen with a dose of 5000µg/ml which is the highest dose of LD-50 in which mice were still alive with minute toxicity.

4.7.2 Measurement of Weight during RA induction and after treatment

Bodyweight was measured on 0,7,14 and 25 days during the procedure of RA induction in mice and after 14 days of treatment. With minute variation, the overall weight of healthy and arthritic mice did not change significantly during RA induction and after treatment with T.ammi extracts and ibuprofen. (Figure 4.21)

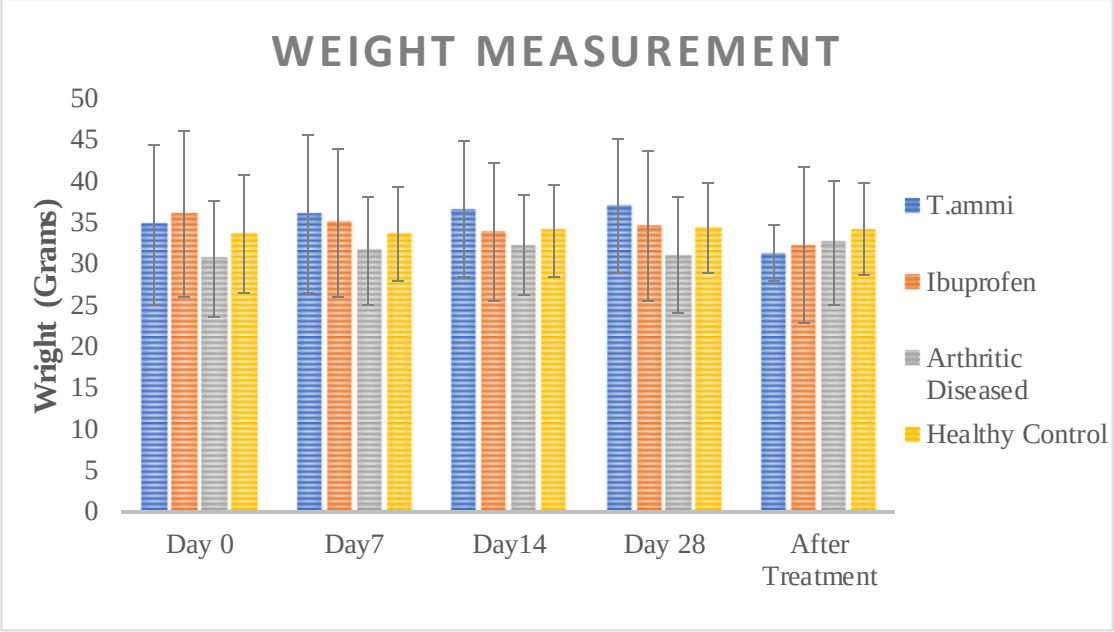


Figure 4.21: Graph depicting no significant increase in weight during RA induction and after treatment.

4.7.3 Measurement of Paw volume during RA induction and after Treatment

Mouse paw was measured by vernier caliper during the period of mouse model project. Paw volume of RA disease model significantly increases ($p < 0.05$). An increase in paw size was

observed in 7 day of treatment and then increase gradually onwards 14 day and 28 days after the second booster dose. (Figure 4.22)

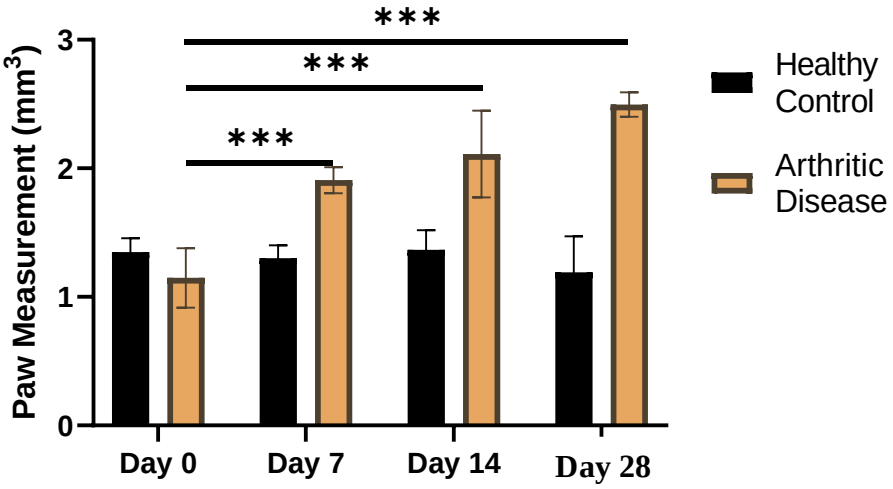


Figure 4.22: Significant increase ($p < 0.001$) in paw volume during RA induction

There was an additional observation raised after 14 days of RA induction which was increased in redness and swelling. A Schematic view of an increase in paw volume was shown in figure 4.23 to which four grade of arthritis was induced in 25 days of RA induction period.

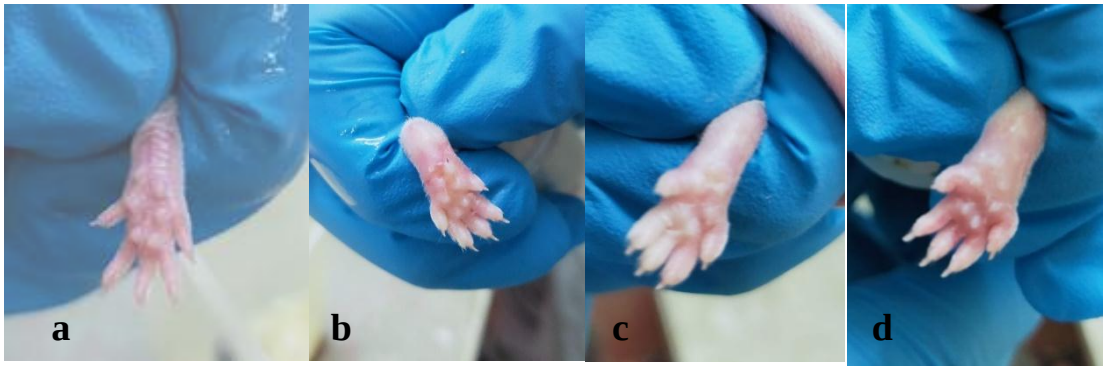


Figure 4.23: Schematic view of paw swelling during RA induction. Grade 1 swelling (a), grade 2 swelling (b), grade 3 swelling (c), grade 4 swelling (d).

Grade 4 arthritic model mice were treated with T.ammi methanolic extracts and ibuprofen with a dose of 5000mg/kg of mouse weight. Mice treated with T.ammi methanolic extract shows constant significant ($p < 0.05$) decrease in paw size depicted revert of Rheumatoid arthritis inflammation (figure 4.24). Results showed that the T.ammi plant had higher anti-

inflammatory properties as compared to the commercialized drug Ibuprofen, T.ammi showed more reduction in paw size inflammation on day 7 and day 14 of treatment.

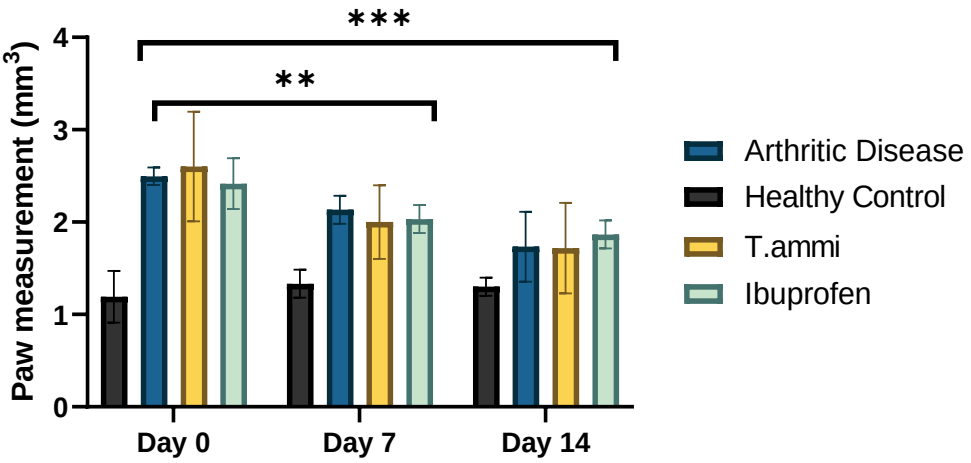


Figure 4.24: Significant (**p < 0.01, ***p<0.001) reduction in paw volume after treatment with T. ammi methanolic extract and ibuprofen.

4.8 Clinical Chemistry

After treatment mice were sacrifices in ASAB Animal house NUST and blood were collected for diagnostic interpretation of LFTs and RFTs. Results were addressed in table 4.9. Significant (p<0.05) difference was observed in between ALT and ALP levels of experimental groups whereas total bilirubin, creatinine, and urea level never changes.

Table 4.9: Significant (p<0.05) difference in Clinical Chemistry parameters of mice in therapeutic analysis. ALT (alanine aminotransferase); ALP (alkaline phosphates); TB (Total Bilirubin); Cr (Creatinine); mg/kg (milligrams per kilogram).

Sr.no	Groups	ALT U/L	ALP U/L	TB mg/dL	Cr mg/dL	Urea mg/dL
1	Healthy	46±9.007	70±7.98	0.4±0.008	0.3±1.8	36±2.69
2	Diseased	73±5.02*	127±2.1*	0.5±1.8	0.28±0.77	33±1.8
3	Ibuprofen	44±3.5	74±7.3	0.5±0.76	0.24±1.48	42±9.4
4	T. ammi	56±4.8*	90±15.84*	0.4±1.02	0.19±0.04	34±10.7

4.9 Evaluation of Catalase Assay

Qualitative

The formation of oxygen bubbles (figure 4.25) when adds hydrogen peroxide into liver homogenate was the confirmation of the presence of extract in the homogenate that causes H2O2 degradation.

Figure 4.25: Qualitative analysis of catalase.

Quantitative

Catalase activity can be determined by measuring the evolution of O_2 or by titrimetrically measuring the reduction of H_2O_2. Catalase activity significantly ($p < 0.05$) increased in T.ammi treated mice as compared to the arthritic model to manifest the outcomes of RA. Higher significant catalase activity can be seen ($p < 0.01$) in healthy control and T.ammi treated mice but there is no significant difference observed in ibuprofen and T.ammi treated groups (figure 4.26). The levels were increased in all extract treated groups compared to the disease-induced group depicting that the extracts have a significant influence on increasing catalase levels (table 4.10)

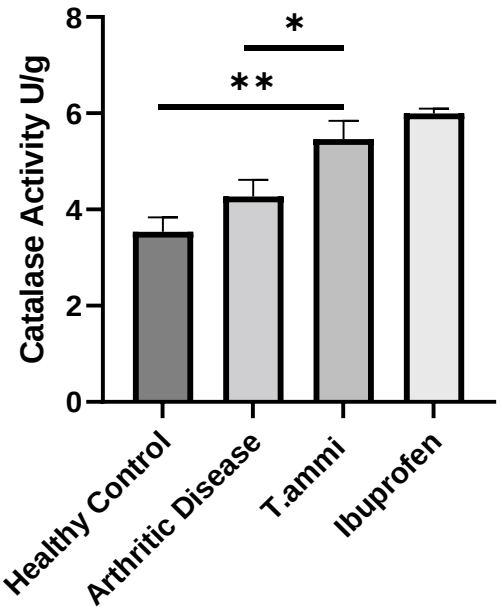


Figure 4.26: Catalase activity of Experimental group depicting significant (* p<0.05 and ** p<0.01) difference in treated compared to disease groups

Table 4.10: Catalase activity of experimental groups in which all the values were expressed in ± SD, n=3, *p<0.05 significant, **p<0.01 highly significant, ***p<0.0001 very highly significant.

Sr. no	Experiment Group	CAT (µM/min/g)
1	Healthy Control	3.53±0.31
2	Arthritic Diseased	4.27±0.35*
3	T.ammi	5.47±0.38**
4	Ibuprofen	6.0±0.10***

4.10 Evaluation of Spleen Indexing

Spleen indexing is done for all the groups i.e., control and treated groups. It was observed that there is a significant increase in the spleen index of the arthritic model and a decrease in the spleen index of T.ammi and ibuprofen treated mice models Decrease in the spleen index revealed decrease in inflammation. (figure 4.27).

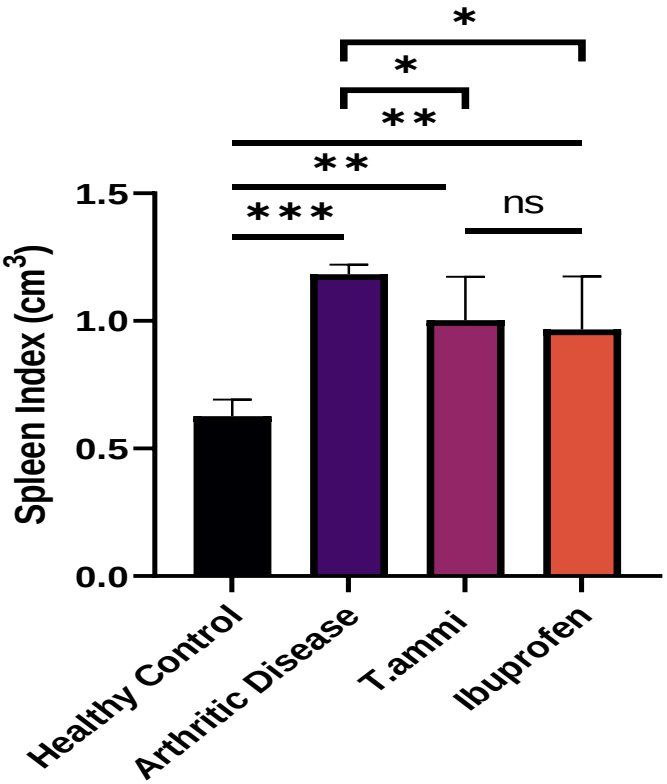


Figure 4.27: Spleen index of the experimental group. *p<0.05 significant, **p<0.01 highly significant, ***p<0.0001 very highly significant.

4.11 Histopathological examination

Histopathological examination was performed on mouse hind paw to examine the destruction in the bone-surrounding areas of CIA paw tissue in parallel to healthy controls. Histopathological analysis of the untreated arthritic group showed bone erosion, cellular infiltration of inflammatory cells, and distorted boundaries in bone tissue ankle joint. This served as the conformation of collagen-induced arthritic in the mice model. however, normal paw histopathological views of mice paws did not show any type of damage in bone tissue and cellular infiltration whereas the arthritic disease group shows a greater extent of bone erosion and cellular infiltration. However, in t.ammi treated and ibuprofen treated arthritic mouse model showed significant restoration in bone denaturation and cellular infiltration depicting a decrease in inflammation. (figure 4.28).

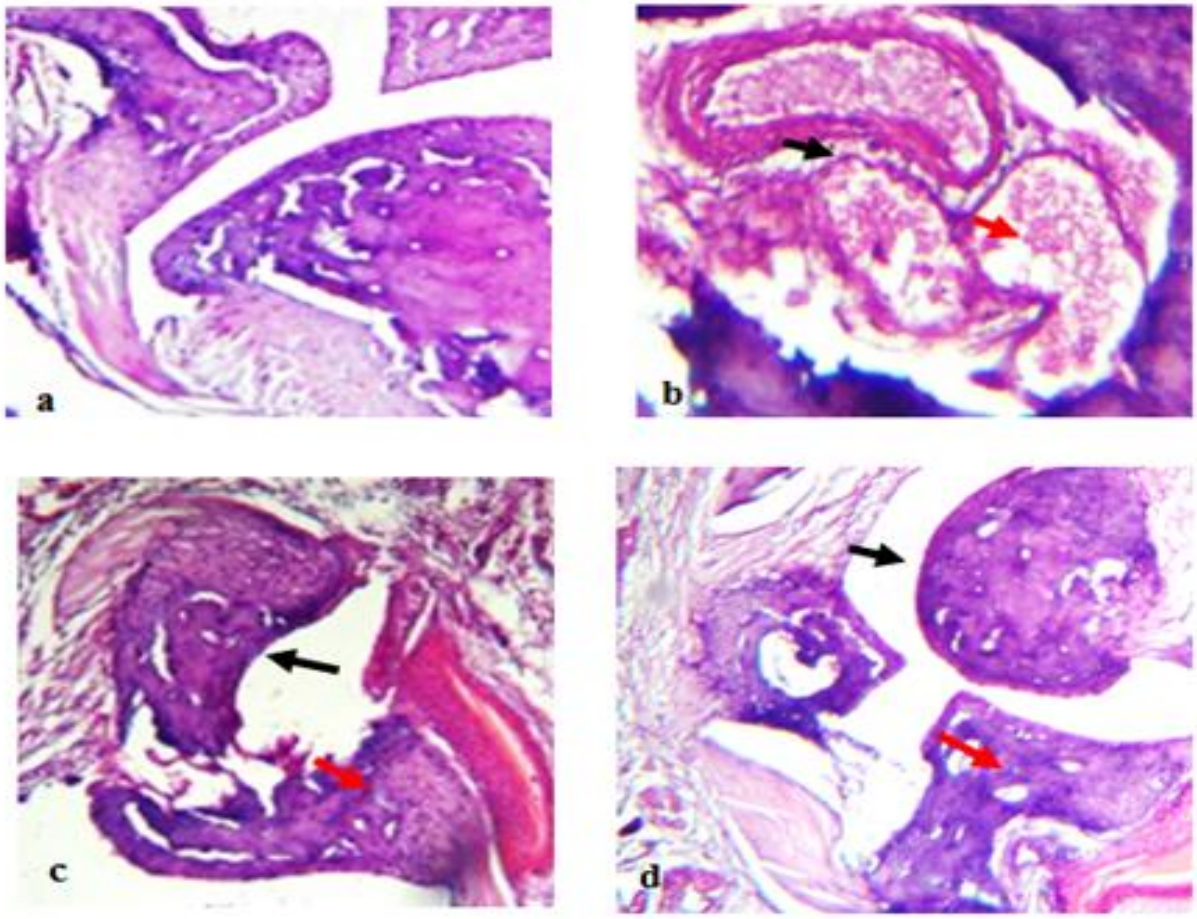


Figure 4.28: Histopathological sections of (a)untreated healthy normal mouse paw, (b) Arthritic diseases mouse paw, (c) T.ammi treated mouse paw, (d) Ibuprofen treated mouse paw. The black arrow indicates bone erosion and the red arrow indicates cellular infiltration.

"In-silico evaluation of Trachyspermum Ammi"

4.12 Evaluation of Drug like properties

933 compounds were found by GC-MS of *Trachyspermum ammi*. They were shortlisted by checking their druglike properties. At the start, compounds were shortlisted based on molecular weight and topological polar surface area (TPSA). Those compounds that have molecular weight out of the range of 150-500 were eliminated which is purely the Lipinski rule. The second elimination level is of TPSA in which compounds that have TPSA greater than 140 were also eliminated. To check the drug likeliness of the remaining 589 compounds they were allowed to be screened by several rules which are Lipinski rule of five, Ghose rule, Muegge rule,

Egan rule, and Veber rule. 270 compounds were shortlisted after being evaluated by swissadme for drug-likeness properties. These compounds were further analyzed by admetSAR for the prediction of ADMET properties absorption, distribution, metabolism, and toxicity evaluation, and after that 94 Compounds are shortlisted. Drug-like properties of finally shortlisted bioactive compounds were listed in Table 4.11 and important parameters for ADMET properties prediction were addressed in Table 4.12.

4.12.1 MOLSOFT

94 compounds were evaluated through the molsoft online tool for the measurement of Blood-brain barrier value which was not in the range of 1.5-2.7. then come 20 compounds that have a blood-brain value greater than 2.7.

4.12.2 BOILED-EGG plot

20 shortlisted compounds from admetSAR were again allowed to screen through BOILED-Egg plot analysis by swissadme through which compounds that were pointed to BOILED-Egg yolk were allowed to cross Blood-Brain Barrier passively and the compounds pointed to white portion were allowed to cross GI track passively. 7 Shortlisted compounds of the present study were lie in a white portion that shows they are not crossed from BBB to CNS seen in figure 4.2. These seven compounds were 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane), Thiosulfuric acid, S-(2-amino-1-phenyl propyl)ester, Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester, 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide, 2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid, Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)-, 6-Oxo-7,11-diazatricyclo[7.3.1.0(2,7)]trideca-2,4-diene-11-carbothioamide. BOILED-Egg plot of these seven compounds we showed in Figure 4.29.

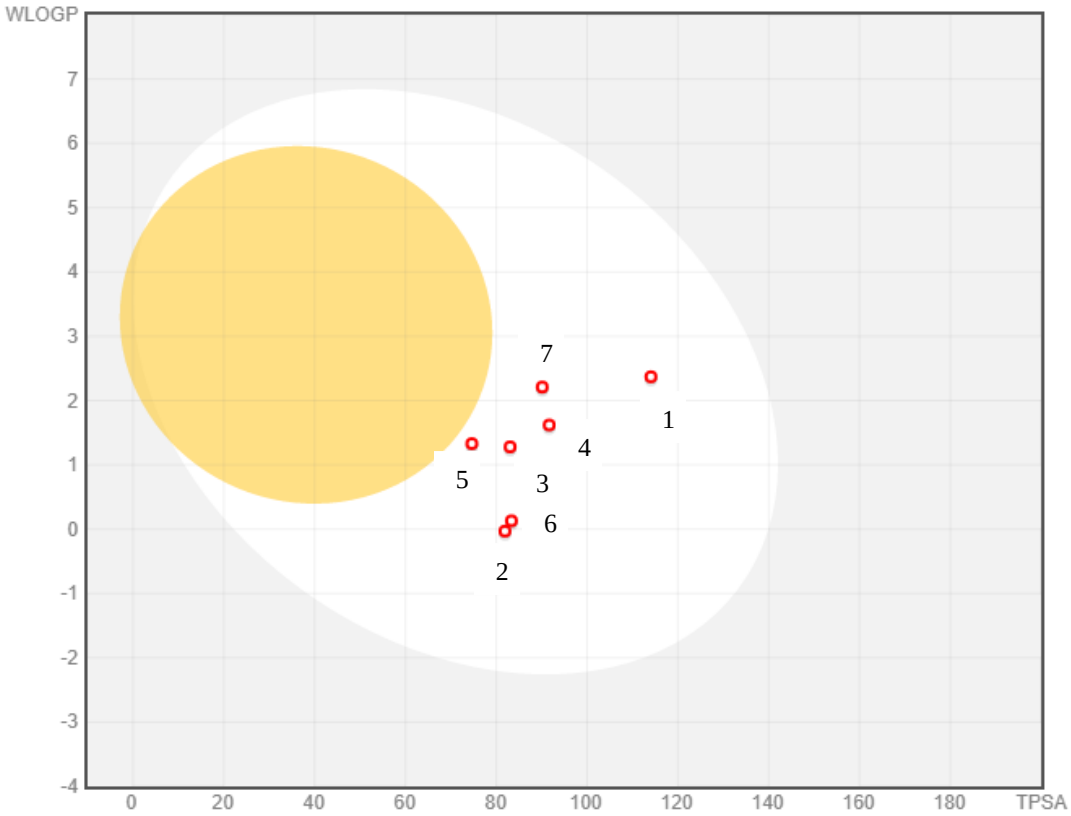


Figure 4.29: BOILED-Egg plot of 7 compounds which shows best drug like properties were; 1) Thiosulfuric acid, S-(2-amino-1-phenylpropyl)ester. 2) Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester. 3) Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester. 4) 4-hydroxy-2-methyl-, 1,1-dioxide, 2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid. 5) Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl). 6) 6-Oxo-7,11-diazatricyclo[7.3.1.0(2,7)]trideca-2,4-diene-11-carbothioamide. 7) 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane)

Table 4.11: Druglike Properties and Blood-Brain Barrier Score of shortlisted 7 compounds (retrieved from Swissadme and Molsoft)

Pubchem ID	560723	575876	547886	611082	534589	542384	46949610
Compound Name	3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane)	Thiosulfuric acid, S-(2-amino-1-phenylpropyl)ester	Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester	2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide	2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid	Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)-	6-Oxo-7,11-diazatricyclo[7.3.1.0(2,7)]trideca-2,4-diene-11-carbothioamide
Molecular Formula	C21H40O8	C9H13NO3S2	C9H15NO4	C9H9NO4S	C12H16O5	C10H10FNO2S	C12H15N3OS
Molecular weight	420.54 g/mol	247.33 g/mol	201.22 g/mol	227.24 g/mol	240.25 g/mol	227.26 g/mol	249.33 g/mol
MlogP	-0.66	0.98	-0.38	0.62	0.72	1.7	1.06
Heavy atoms	29	15	14	6	17	15	17
Rotatable bonds	2	4	5	0	4	2	1
HBA	8	4	4	4	5	4	1
HBD	0	2	1	1	2	2	1
TPSA	73.84 Å²	114.07 Å²	81.92 Å²	83.06 Å²	91.67 Å²	74.63 Å²	83.35 Å²
Molar Refractivity	107.25	61.86	48.62	55.49	60.69	59.75	74.05
Lipinski Violation	No	No	No	No	No	No	No

BBB score	2.97	3.35	3.15	2.88	3.33	3.18	3.64

Table 4.12: ADMET properties of drug-like bioactive compounds

Parameters	Ideal	560723	575876	547886	611082	534589	542384	46949610
Absorption								
Blood brain barrier	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
Human Intestinal Absorbtion	+ive	+ive	+ive	+ive	+ive	+ive	+ive	+ive
Human Oral Bioavailability	+ive	+ive	+ive	+ive	+ive	+ive	+ive	+ive
Caco2 Permeability	+ive	+ive	+ive	+ive	+ive	+ive	+ive	+ive
P glycoprotein Substrate	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
Metabolism								
CYP450 2C9 Susctrate	+ive	-ive	-ive	-ive	+ive	-ive	+ive	-ive
CYP450 2D6 Substrate	+ive	-ive	+ive	-ive	-ive	-ive	-ive	-ive
CYP450 3A4 Substrate	+ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
CYP450 1A2 Inhibitor	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
CYP450 2C9 Inhibitor	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
CYP450 2D6 Inhibitor	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
CYP450 2C19 Inhibitor	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive

CYP450 3A4 Inhibitor	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
Toxicity								
AMES Toxicity	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
Human Ether-ago-go-Related Gene Inhibition	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
AMES mutagenesis	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
Carcinogens	-ive	+ive	-ive	-ive	-ive	-ive	-ive	-ive
Hepatotoxicity	-ive	-ive	-ive	-ive	-ive	-ive	-ive	-ive
Acute Oral Toxicity	III/IV	III	III	III	III	III	III	III

4.13 Therapeutic Targets of Rheumatoid Arthritis

409 total targets of Rheumatoid arthritis were retrieved from many sources in which 227 were the literature reviewed targets, 117 targets from the therapeutic target database (TTD), and 65 targets from the Comparative toxicogenomics database (CTD).

4.14 Prediction of targets of T.ammi shortlisted compounds

Targets of shortlisted compounds were retrieved from swiss target prediction and CTD. Several targets with their respective compounds are as follows and also in table 4.4. 2-dimensional structures of shortlisted compounds were retrieved from PubChem shown in figure 4.30.

1. 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane) had **112** targets.
2. Thiosulfuric acid, S-(2-amino-1-phenylpropyl)ester had **110** targets.
3. Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester had **110** targets.
4. 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide had **106** targets.

5. 2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid had **129** targets.
6. Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl) had **112** targets.
7. 6-Oxo-7,11-diazatricyclo [7.3.1.0(2,7)] trideca-2,4-diene-11-carbothioamide had **114** targets.

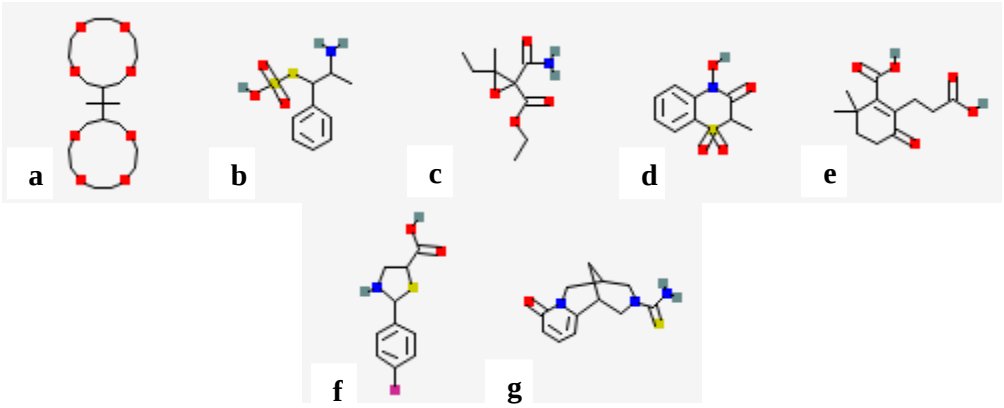


Figure 4.30: 2D structures of bioactive compounds. (a) 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane), (b) Thiosulfuric acid, S-(2-amino-1-phenylpropyl)ester (c) Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester, (d) 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide, (e) 2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid , (f) Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl), (g) 6-Oxo-7,11-diazatricyclo [7.3.1.0(2,7)] trideca-2,4-diene-11-carbothioamide.

4.15 Network Construction

BISOGENET PPI network of RA targets was made in Cytoscape containing 378 nodes and 1275 edges or interactions. T.ammi compounds targets were uploaded to STRING separately and then send to the Cytoscape where further analysis was done. After that intersection merged networks of the targets of compounds and RA were made in which common targets between the compounds and disease were obtained that contains a smaller number of targets as in original compound targets obtained from databases (table 4.13). The networks contained nodes and edges of the compound target network were also illustrated in table 4.13. Some of the targets were omitted from Cytoscape to be judged and they were removed from the network. Then total Cytoscape merged targets of seven compounds counts 98.

A combined network of all seven compounds and targets of the respective compounds were made that linked to Rheumatoid Arthritis (figure 4.31).61 targets were retrieved that were common in both RA and seven compounds. While entering data in Cytoscape compounds were labeled as nodes and targets were labeled as node targets. C-T network separate proteins that are common in all the seven compounds which are TLR9, IDO1, KDR, JAK2, PTGS2, ALOX15, JAK1, ALOX5, TYK2.

Functions of all proteins were confirmed through uniport which was displayed in a figure (4.32). The graph illustrated that from 98 merged proteins many proteins lie in the categories of kinases, oxidoreductases, and DNA binding proteins.

Table 4.13: Targets of all seven compounds obtained from the databases and detail of their Cytoscape networks, the union merged targets of Rheumatoid arthritis and disease obtained from Cytoscape.

Compounds	Targets of compounds from STP, CTD, and Target Hunter	Cytoscape merged targets (Rheumatoid Arthritis and compounds)
3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane)	112 Nodes: 109 Edges: 737	16 Nodes: 16 Edges: 36
Thiosulfuric acid, S-(2-amino-1-phenylpropyl)ester	110 Nodes:109 Edges:623	13 Nodes: 13 Edges: 27
Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester	110 Nodes: 109 Edges: 493	16 Nodes: 16 Edges: 41
2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide	106 Nodes: 96 Edges: 486	14 Nodes: 14 Edges: 50
2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-	129 Nodes: 123	11 Nodes: 11

enecarboxylic acid	Edges: 507	Edges: 21
Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl),	112 Nodes: 105 Edges: 387	7 Nodes: 7 Edges: 13
6-Oxo-7,11-diazatricyclo [7.3.1.0(2,7)] trideca-2,4-diene-11-carbothioamide.	114 Nodes: 103 Edges: 347	21 Nodes: 21 Edges: 52

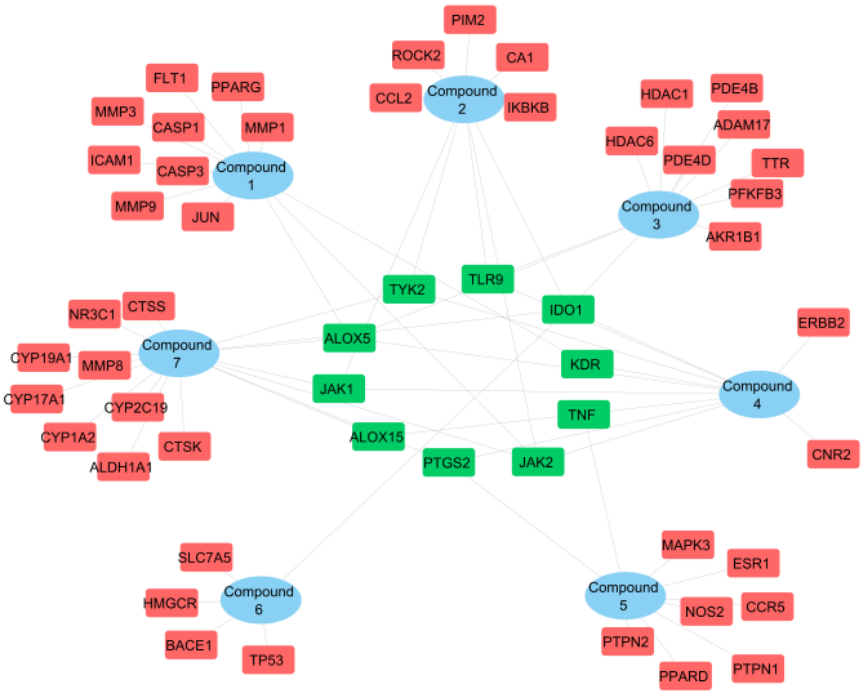


Figure 4.31: Compound target network that illustrates common targets between seven compounds linked to RA. Blue eclipse shape illustrates compounds, red rectangle shapes are the individual targets of seven compounds and green rectangles were the common targets of the compounds. Compounds were labeled as **Compound 1** (3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane)), **Compound 2** (Thiosulfuric acid, S-(2-amino-1-phenyl propyl)ester), **Compound 3** (Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester), **Compound 4** (2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide), **Compound 5** (2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid) , **Compound 6**

(Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)), **Compound 7** (6-Oxo-7,11-diazatricyclotrideca-2,4-diene-11-carbothioamide).

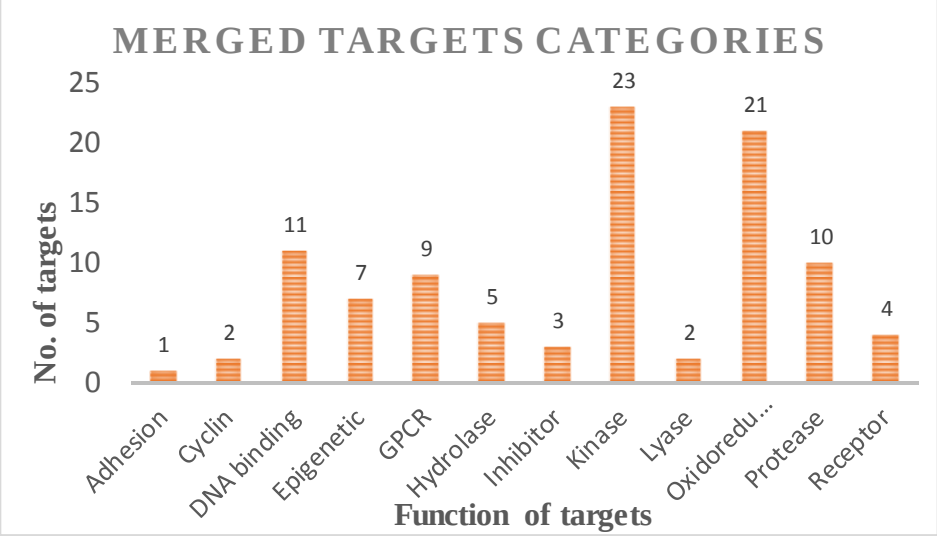
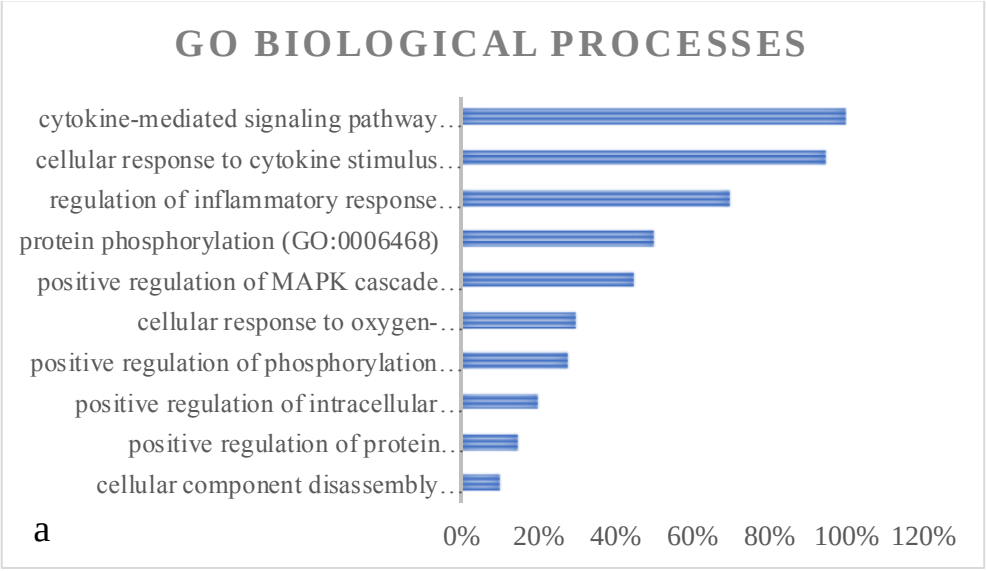
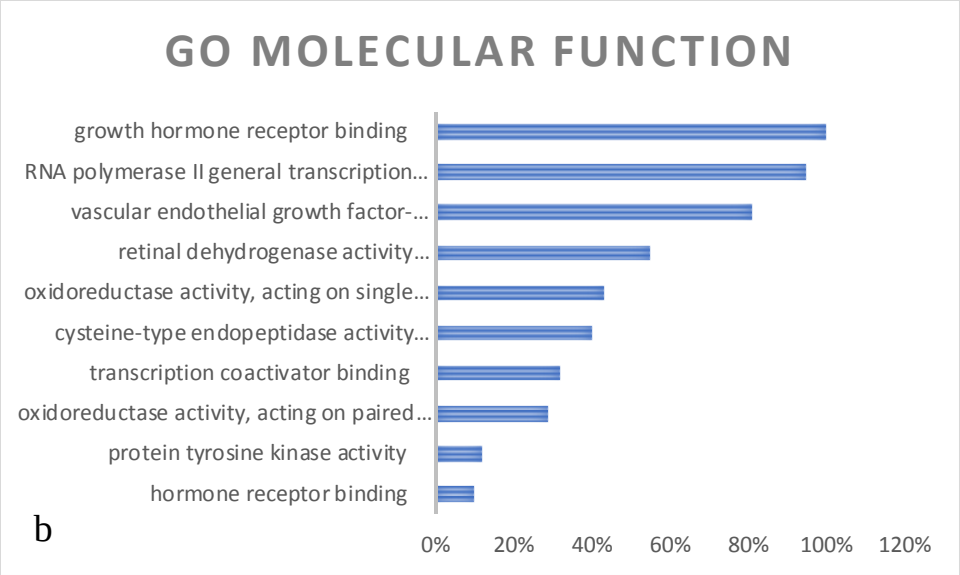


Figure 4.32: Merged target categories.

4.16 Enrichment and Pathway Analysis

Enrichment analysis was performed for 61 common targets of seven compounds and RA. GO analysis consisted of biological process (BP), cellular component (CC), and molecular function (MF). As showed in Figure 4.33 (a,b,c), the top 10 enrichment terms are visualized by the bar plot diagram. Based on $p < 0.05$ value.





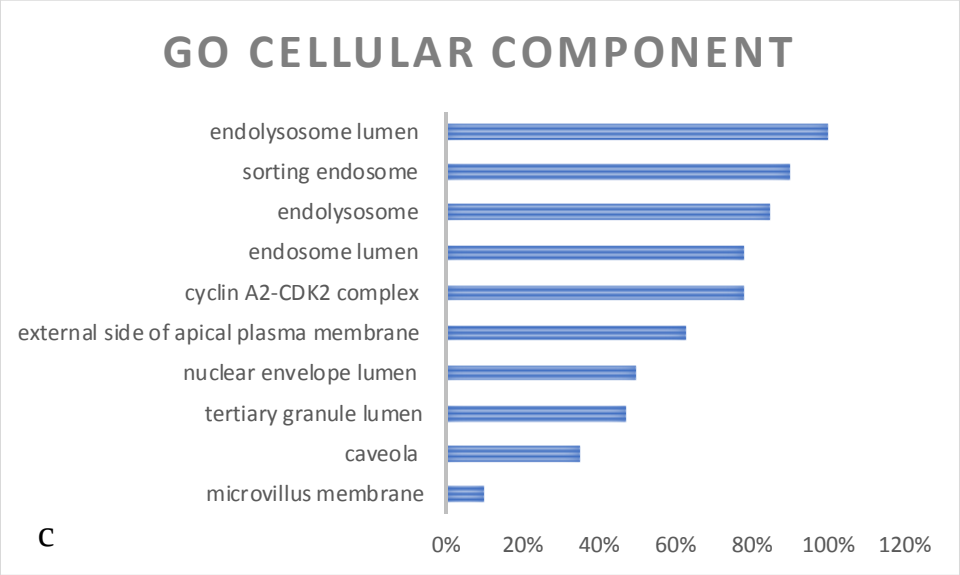


Figure 4.33: GO analysis of targets, the top 10 significant enrichment terms in BP (a), CC (b), and MF (c).

4.17 KEGG Enrichment Analysis

The KEGG pathways was employed to investigate the purpose and indicating pathways of the predicted target genes, with the top 10 of the prospective pathways envisioned with the pathways-targets-compounds network (figure 4.34). The outcomes demonstrated that various targets are coupled with potential signaling pathways which are linked with the inception and pathogenesis of RA (Table 4.14).

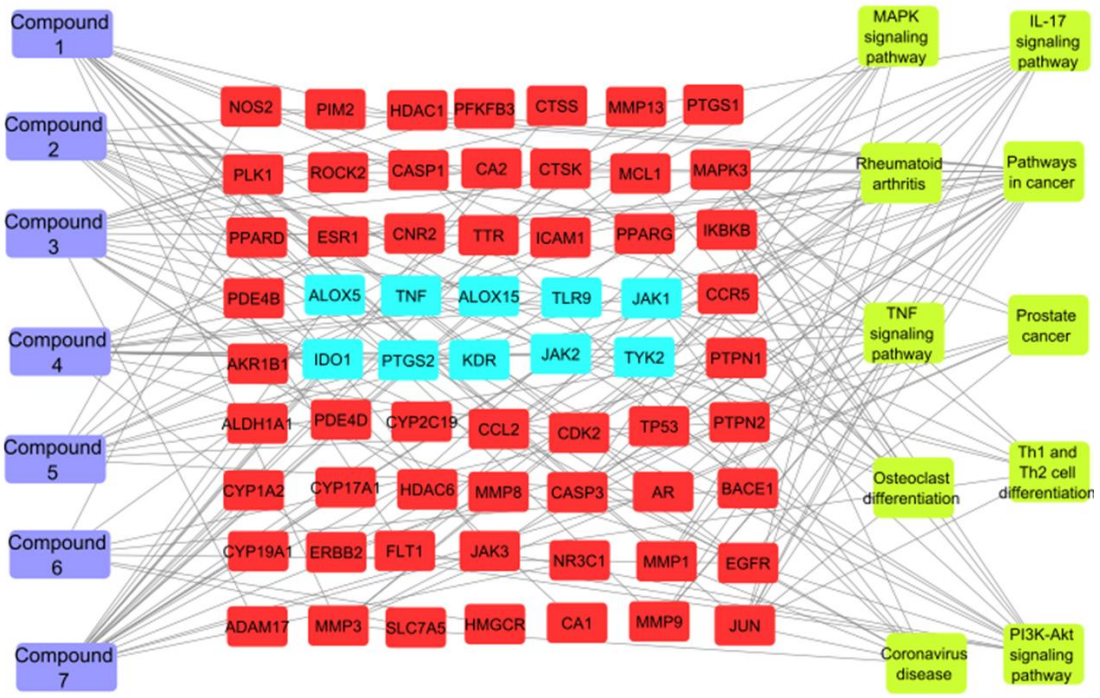


Figure 4.34: Compound-target-pathway network. Purple square boxes were the seven compounds, red square boxes were the targets of all 7 seven compounds, blue boxes were the common targets of the seven compounds and green boxes were the interacting KEGG pathways.

Table 4.14: Significant ($p<0.05$) enriched pathway of the merged targets and targets included in respective pathway.

Pathway	P value < 0.05	Targets
Pathways in cancer	7.70E-20	JUN, NOS2, MMP1, HDAC1, ROCK2, PTGS2, MMP9, ESR1, EDFR, IKBKB, AR, CASP3, ERBB2, CGK2, PPARG, PIM2, JAK2, JAK3, TP53, JAK1, PPARD, MAPK3
IL-17 signaling pathway	3.83E-15	IKBKB, JUN, MMP13, MMP1, CASP3, MMP3, CCL2, PTGS2, TNF, MMP9, MAPK3
TNF signaling pathway	1.20E-12	IKBKB, JUN, CASP3, MMP3, CCL2, PTGS2, TNF, MMP9, ICAM1, MARK3
Coronavirus disease	3.82E-12	IKBKB, ADAM17, JUN, MMP1, MMP3, CASP1, CCL2, TYK2, TNF, EGFR, JAK1
Prostate cancer	1.23E-11	IKBKB, AR, MMP3, CDK2, MMP9, ERBB2, TP53, EGFR,

		MAPK3
Rheumatoid arthritis	3.38E-10	JUN, FLT1, MMP1, CTSK, MMP3, CCL2, TNF, ICAM1
PI3K-Akt signaling pathway	5.12E-10	IKBKB, FLT1, ERBB2, CDK2, KDR, JAK2, JAK3, TP53, EGFR, JAK1, MAPK3, MCL1
Osteoclast differentiation	4.11E-09	IKBKB, JUN, CTSK, PPARG, TYK2, TNF, JAK1, MAPK3
Th1 and Th2 cell differentiation	1.09E-08	IKBKB, JUN, TYK2, JAK2, JAK3, JAK1, MAPK3
MAPK signaling pathway	1.59E-08	IKBKB, JUN, FLT1, CASP3, ERBB2, KDR, TNF, TP53, EGFR, MAPK3

4.18 Docking Analysis

Docking is performed by PyRx of all compounds and their merged targets (table 4.15). 3D/PDB structures of proteins were downloaded from protein databank and three dimentional structures of bioactive constituents were copied from PubChem in SDF format. PDB file of TLR9 was not available at the protein data bank so it was excluded from the docking analysis initially. Compounds with their targets were mentioned in table 4.6 but **2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide** linked to all 10 merged targets of RA and compounds and also gave higher docking score binding energies with TNF, PTGS2, and TYK2 as compared to the same compound. **3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane)** also gave higher binding energy with the only ALOX5 from all of its targets. Docking results (table 4.15) represented higher binding energies of 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane) with ALOX5 target and 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide compound with TNF, PTGS2 and TYK2. Docking was visualized by Discovery studio and docking images having higher docking scores were shown in (figure 4.35-4.38). Docking parameters like hydrogen bond interaction, pi-anion, pi-alkyl, pi-sigma interactions. Interacting atoms, bonding atoms, bond length, bond category, and bond type of 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane) were enlisted in table 4.16 and of 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide in table 4.17.

Table 4.15: Compound and their merged targets with binding energies.

Compound	SDF files	Targets	Binding Energies
3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane)	CID-560723	KDR	-6.2
		JAK2	-6.8
		ALOX5	-7.9
Thiosulfuric acid, S-(2-amino-1-phenyl propyl) ester	CID-575876	JAK2	-5.3
		IDO1	-5.8
		TYK2	-5.9
		JAK1	-5.4
Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester	CID-547886	ALOX5	-5.2
		IDO1	-5
2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide	CID-611082	KDR	-5.3
		JAK2	-5.6
		TNF	-7.2
		ALOX5	-6.9
		ALOX15	-6.5
		PTGS2	-7.9
		IDO1	-6.7
		TYK2	-7.4
		JAK1	-6.2
2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid	CID-534589	TNF	-6.8
		PTGS2	-5.8
Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)-	CID-542384	1DO1	-6.9
6-Oxo-7,11-diazatricyclo [7.3.1.0(2,7)]trideca-2,4-diene-11-carbothioamide	CID-46949610	JAK2	-5.4
		ALOX5	-6.5
		ALOX15	-6.1
		PTGS2	-6
		IDO1	-5.8
		TYK2	-6.5
		JAK1	-5.7

Table 4.16: Interacting atoms, bonding atoms, bond length, bond category, and bond type of ALOX5 with its ligand 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane).

Targets	Binding score	Interacting atoms	Bond length	Bond Category	Bond type
ALOX5	-7.9	ARG (A:101)	2.61302	Hydrogen Bond	Conventional Hydrogen Bond
		GLU (A:134)	3.68027	Hydrogen Bond	Carbon Hydrogen Bond
		ASP (A:166)	3.49149	Hydrogen Bond	Carbon Hydrogen Bond
			3.61606	Hydrogen Bond	Carbon Hydrogen Bond
		GLN (A:141)	3.50101	Hydrogen Bond	Carbon Hydrogen Bond
		PRO (A:164)	5.0766	Hydrophobic	Alkyl
		LEU (A:163)	3.55121	Hydrogen Bond	Carbon Hydrogen Bond
		ASP (A:162)	3.64076	Hydrogen Bond	Carbon Hydrogen Bond

Table 4.17: Interacting atoms, bonding atoms, bond length, bond category, and bond type of TNF, PTGS2, and TYK2 with their ligand 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide.

Targets	Binding score	Interacting atoms	Bond length	Bond Category	Bond type
TNF	-7.2	GLN: B102	2.71803	Hydrogen Bond	Conventional Hydrogen Bond
		GLN: B102	2.54839	Hydrogen Bond	Conventional Hydrogen Bond
		GLN: A102	2.04164	Hydrogen Bond	Conventional Hydrogen Bond
		GLU: B116	2.04164	Electrostatic	Pi-Anion
PTGS2	-7.9	HIS (A:374)	3.07705	Hydrogen Bond	Conventional Hydrogen Bond
		ALA (A:185)	4.88825	Hydrophobic	Pi-Alkyl
		LEU (A:377)	3.8438	Hydrophobic	Pi-Sigma
		LEU (A:376)	5.39671	Hydrophobic	Pi-Alkyl
		GLN (A:189)	3.9919	Hydrophobic	Pi-Sigma
		TRP (A:373)	2.45824	Hydrogen	Conventional Hydrogen Bond

				Bond	
		HIS (A:374)	2.6179	Hydrogen Bond	Conventional Hydrogen Bond
			3.07705	Hydrogen Bond	Conventional Hydrogen Bond
TYK2	-7.4	TRP (A:1067)	3.61068	Hydrophobic	Pi-Sigma
		SER (A:1063)	4.25088	Hydrophobic	Amide-Pi Stacked
		PRO (A:1064)	4.22338	Hydrophobic	Pi-Alkyl
		ASN (A:1023)	2.37444	Hydrogen Bond	Conventional Hydrogen Bond
		ASN (A:1028)	2.36289	Hydrogen Bond	Conventional Hydrogen Bond
			2.33773	Hydrogen Bond	Conventional Hydrogen Bond
		ARG (A:1027)	2.55688	Hydrogen Bond	Conventional Hydrogen Bond

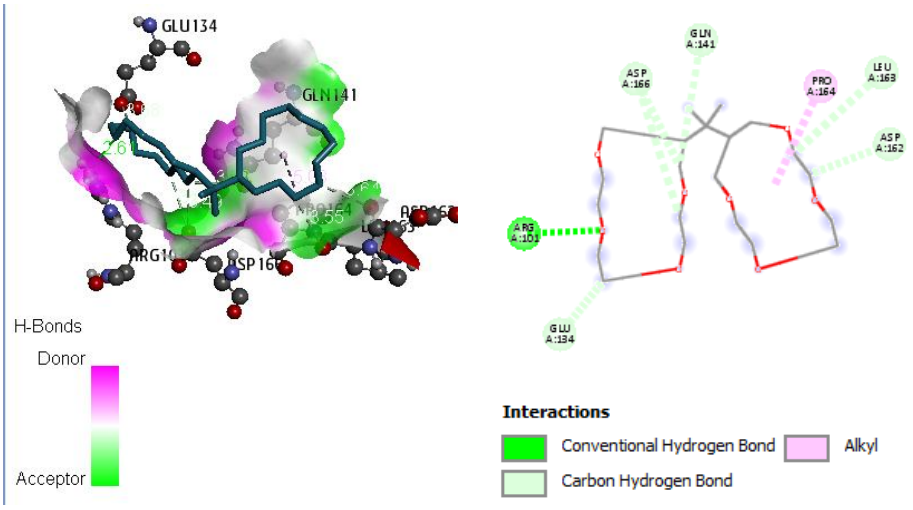


Figure 4.35: Docking of ALOX5 with 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane). Docking image (left), Discovery studio visualization (right).

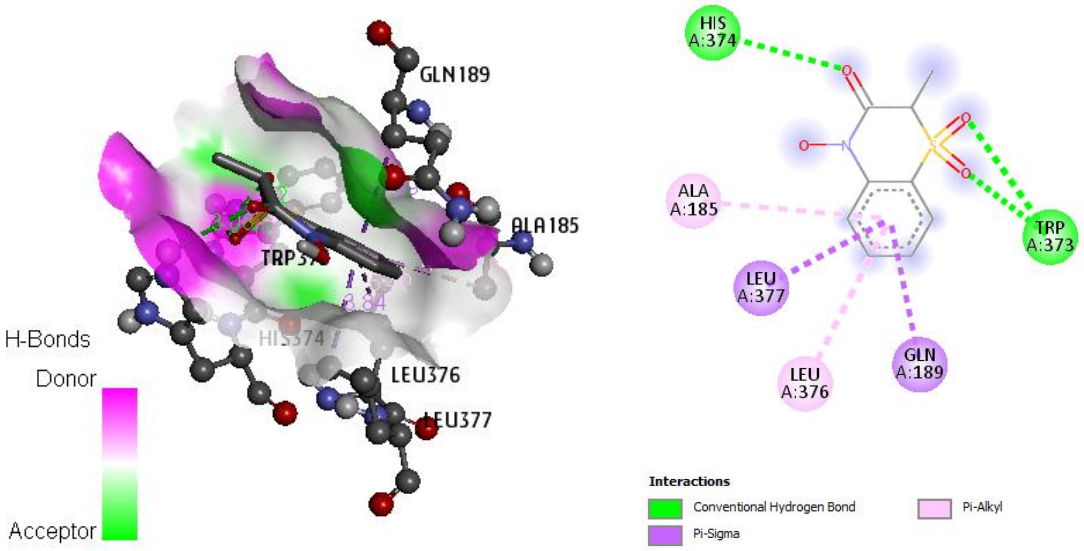


Figure 4.36: Docking of PTGS2 with 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide. Docking image (left), Discovery studio visualization (right).

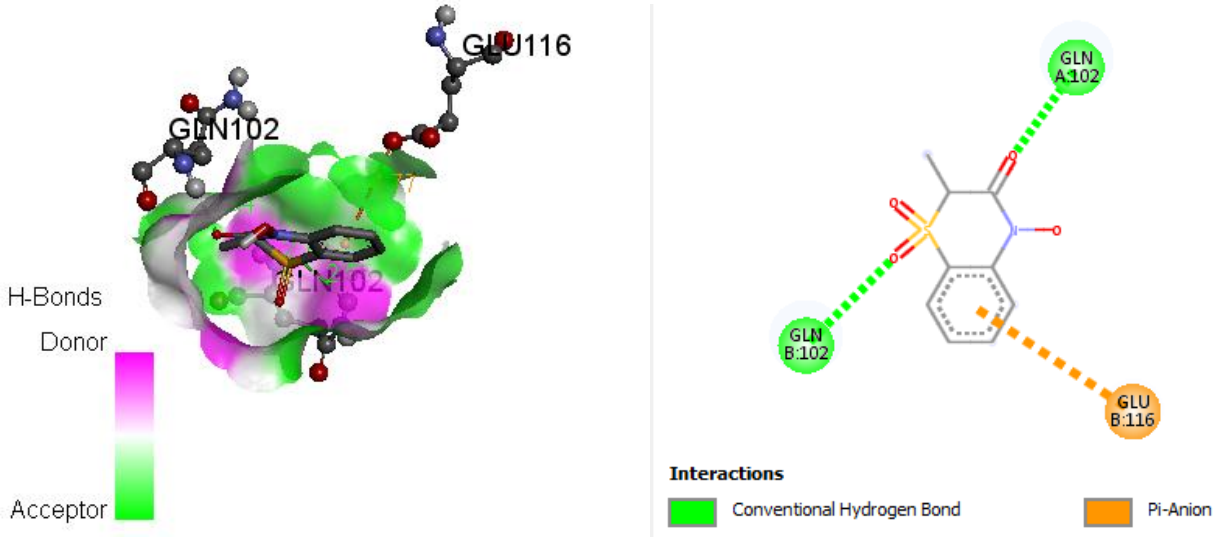


Figure 4.37: Docking of TNF with 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide. Docking image (left), Discovery studio visualization (right).

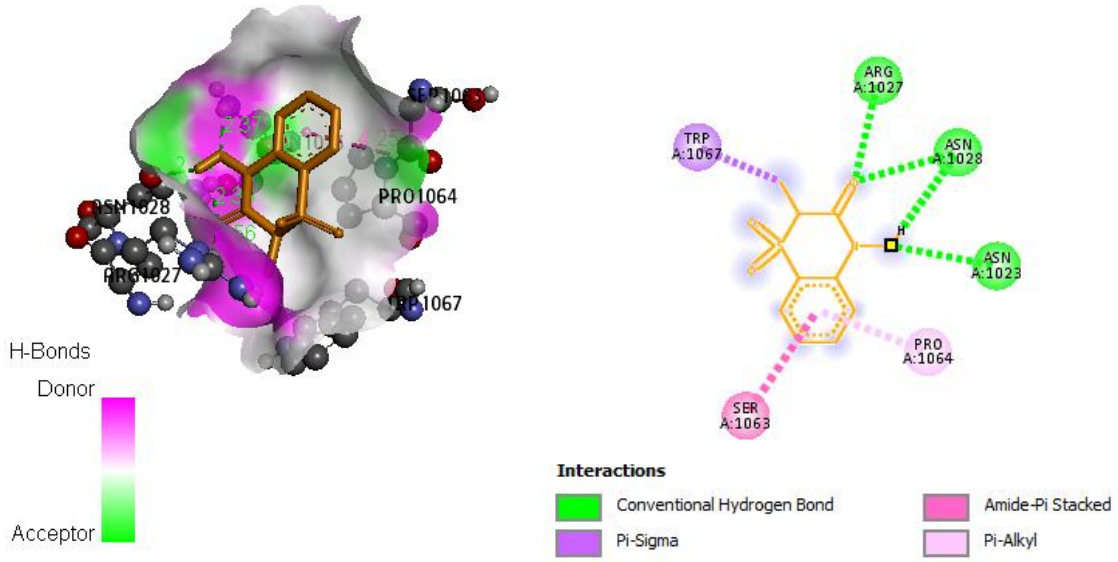


Figure 4.38: Docking of TYK2 with 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide. Docking image (left), Discovery studio visualization (right).

After docking of respective targets to their ligands, binding sites of the ligand or bioactive compounds were analyzed by UNIPROT so that it can be evaluated, at which site ligand docked at protein target which was enlisted in table 4.18.

Table 4.18: Matching of Docked ligands residues with the residues of targets functional domain.

Targets	Functional Domain	Number of Residues	Residues at which Ligand docked
ALOX5	PLAT domain Lipoxygenase Domain	2-118 119-674	ARG (A:101) GLU (A:134) ASP (A:166) GLN (A:141) PRO (A:164) LEU (A:163) ASP (A:162)
TNF	C domain 1	88-233	GLN: B102 GLN: B102 GLN: A102 GLU: B116

PTGS2	Peroxidase Site	97-598	HIS (A:374) ALA (A:185) LEU (A:377) LEU (A:376) GLN (A:189) TRP (A:373) HIS (A:374)
TYK2	Protein kinase domain 2	897-1176	TRP (A:1067) SER (A:1063) PRO (A:1064) ASN (A:1023) ASN (A:1028) ARG (A:1027)

Discussion

Rheumatoid Arthritis is a chronic, autoimmune, and inflammatory degenerative disease leading to synovial joint and cartilage destruction and bone erosion most commonly in women. Several pathways had key regulatory roles in the pathogenesis and progression of RA that mainly involves TNF signaling cascade, IL-1, IL-6, IL-17 signaling pathway. (Noack & Miossec, 2017.) Much traditional allopathic medication such as non-steroidal anti-inflammatory drugs (NSAIDs), Disease-modifying anti-rheumatic drugs (DMARDs), corticosteroids, and several analgesics reducing the inflammation and bone deformities caused by RA but also had many side effects like gastrointestinal problems, Liver problems, central nervous system problems, and hypertension. Besides these side effects, scientists move towards herbal medicine because of its cheaper availability, less cytotoxic effects, economical and have anti-inflammatory, anti-rheumatic, anti-viral, and antioxidant properties. (Nimesh, 2018). *Trachyspermum Ammi* was the plant under study to which bioactive compounds were shortlisted and their druglike activities were predicted in-silico.

First of all methanolic and aqueous extracts were prepared by maceration, filtration, and drying. (Hammash et al., 1970). Phytochemical screening was done quantitatively by colorimetric assays in which the presence of phenols, flavonoids, alkaloids, coumarins, diterpenes, triterpenes, tannins, and cardiac glycosides was illustrated. (Hossein & Sakha, 2015) whereas quantitative phytochemical screening was carried out by Total phenolic content and total flavonoid content. The assistance of selecting these two contents was due to their functional groups that had scavenging properties. TPC revealed that the concentration of phenols was greater in methanolic extracts as compared to aqueous extracts. TFC revealed that the concentration of flavonoids was greater in methanolic as compared to aqueous extracts. These results illustrated that the methanolic extract of T.ammi has many bioactive compounds that have radical scavenging properties to reduce reactive oxygen species (ROS) species which are elevated in Rheumatoid Arthritis. The data of total flavonoids and total phenolic of polyherbal formulation were expressed as mg of quercetin and gallic acid equivalents respectively/ 100 g of dry mass (Kamtekar et al., 2014).

Further T.ammi bio profiling manifested by its antioxidant potential assess by 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Chitme et al., 2016) and Hydrogen peroxide radical scavenging assay. (Bhatti et al., 2015) by using ascorbic acid and ibuprofen as a standard. In both the assays free radicals were given by DPPH and hydrogen peroxide respectively acting as oxidized species that are scavenged or reduced by the T.ammi aqueous and methanolic extracts. Due to this, the reducing level of ROS species causes an increase in the % inhibition of the extracts. Results reported that T.ammi methanolic extract had a greater % inhibition potential of antioxidant activity in both DPPH and hydrogen peroxide radical scavenging assay as compared to t.ammi aqueous as well as standard ascorbic and commercial drug ibuprofen.

The anti-inflammatory property of T.ammi was proved by Protein denaturation inhibition potential. Protein was degraded at high temperature but due to the anti-inflammatory activity of the T.ammi, it prevents the albumin protein from denaturation at $57^{o}C$. % inhibition potential of extracts, as well as standard ascorbic acid and ibuprofen, increases with an increase in concentration. But T.ammi methanolic extract possesses greater % inhibition potential as compared to T.ammi aqueous, ascorbic acid, and ibuprofen (Alamgeer et al., 2015).

By considering all these assays it was illustrated that T.ammi methanolic extracts had better antioxidant, anti-inflammatory, and concentration of phenolics and flavonoids. So this methanolic extract was sent to USPCASE for GC-MS analysis and generation of a compound library.

Toxicity analysis of T.ammi was carried out in a mouse model at which different doses were administered to the mice by the enegide method. Signs of toxicity were measured after 1 hour, 4 hours and 24 hours of drug administration and there were no signs of toxicity were observed in the mouse model under the course of the experiment. All the mice used under this study were alive without any toxicity measured. (Chinedu et al., 2013).

The animal mouse model was constructed by Collagenase and frauds adjuvant injection administration. Type II Collagenase protein in the CIA model was used for cartilage destruction whereas bovine serum albumin and frauds adjuvant had the properties to cause inflammation and angiogenesis. A significant increase in paw size was observed after 28 days of injection administration followed by 14 days of treatment with T.ammi extract and ibuprofen a

commercial drug that had reported anti-inflammatory properties. After 14 days of treatment, there is a significant reduction in mouse paw and edema which illustrates minimize in inflammation in mouse paw. (Qamar et al., 2020).

Clinical Chemistry of mouse model such as liver function tests and renal function tests were performed to illustrates the further toxicity to mouse liver and kidney by T.ammi extract. ALT, ALP, total bilirubin were performed under LFTs whereas Urea and creatinine were performed under RFTs. The study reported an increase in the level of ALT and ALP in the arthritic disease model whereas significantly reduced in the T.ammi treated and Ibuprofen treated groups. These results assumed that the liberated liver function tests due to the production of reactive oxygen species in the liver and kidney were restored to some extent with the help of t.ammi treatment which possesses that T.ammi had anti-inflammatory properties. (Dash et al., 2007) (Sher & Hung, 2013).

Anti-inflammatory properties of T.ammi were also presumed by the Catalse assay of the liver. Catalase activity is increased in arthritis because catalase function is to denature or inhibit the ROS species produced in arthritis. By administering T.ammi and Ibuprofen as a treatment in the CIA mouse model illustrated that the antioxidant nature of T.ammi could reduce the radical oxygen species in the arthritic mouse model. So, in the experimental group increase in the catalase activity as compared to the normal group showed an increase in the reduction of ROS and reduction in inflammation. (Dassprakash et al., 2012)

Spleen indices were performed to examine the leukocyte breakdown level due to inflammation. Normal spleen function was to deteriorate the red blood cells but in arthritic inflammation, spleen goes to breakdown white blood cells greater as compared to red blood cells which cause storage of WBCs in the spleen leads to spleen enlargement. In the experimental group, the arthritic disease has a significantly enlarged spleen but the size of the spleen is reduced in T.ammi and Ibuprofen treated mice model which predicts the reduction in inflammation. (Li et al., 2017).

Histopathology of four experimental groups was carried out which includes normal healthy mice, arthritic diseased group, T.ammi treated group, and ibuprofen treated groups. In normal mice, no bone erosion and synovial cellular Infiltration were seen in them but these are

significantly observed in the arthritic disease model. In the T.ammi treated model and ibuprofen treated model, the level of bone erosion and cellular infiltration reduces to extant which depicts reduction in inflammation.

After assuming the anti-inflammatory and anti-arthritic activity of T.ammi, we had to locate the specific compounds which are responsible for these activities. For that purpose network pharmacology approach was used which started from the GCMS data library of T.ammi methanolic extracts. Compounds were shortlisted by using the drug-likeness prediction rules which includes Lipinski rule of five (ROF) parameters proposed by (Lipinski et al., 1997), ghoose rule (Arup K. Ghose et al., 1998), Veber rule (Daniel F. Veber et al., 2002), Egan rule (William J. Egan et al., 2000), Muegge rule (Ingo Muegge et al., 2001). Important noticeable parameters for these rules were the molecular weight ranges between 150-500 daltons, TPSA ≤ 140 A, rotatable bonds ≤ 15, and a number of hydrogen bond donors and acceptor atom ranges from 5-10. After filtering from these rules remaining compounds were subjected to Molsoft for blood-brain barrier prediction and the compounds which come in a range of 1.5-2.7 were considered as druglike compounds because compound values greater than 2.7 assumes to cross the blood-brain barrier. Finally, the BOILED Egg plot was made by swiss adme and the compounds that existed in the yellow and grey portion are eliminated because these compounds have the ability to cross the blood-brain barrier. Only compounds that were present in the white portion were selected for further processing because the compounds that lie in this area had the greater GIT permeability. (Daina & Zoete, 2016).

Seven compounds were shortlisted by all these procedures that have significant antioxidant and anti-inflammatory properties which were 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane), Thiosulfuric acid, S-(2-amino-1-phenyl propyl)ester, Oxirane-2-carboxylic acid, 2-aminocarbonyl-3-ethyl-3-methyl-, ethyl ester, 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide, 2-(2-Carboxyethyl)-6,6-dimethyl-3-oxocyclohex-1-enecarboxylic acid, Thiazolidine-5-carboxylic acid, 2-(4-fluorophenyl)-, 6-Oxo-7,11-diazatricyclo[7.3.1.0(2,7)]trideca-2,4-diene-11-carbothioamide. ADMET properties of these druglike compounds again proposed their drug-like efficacy.

Top regulatory targets of RA and compounds were retrieved from different databases and allowed to form protein-protein interaction network construction via STRING (Confidence level 4 for the RA disease network and Confidence level 9 for compound targets network) and from that networks of compounds and disease targets were exported to Cytoscape. By using the merge tool in Cytoscape the disease compound merged target was generated which illustrated the most dominant protein targets in both the disease and RA. Enrichment Analysis was done by and KEGG pathways were retrieved that involve the pathogenesis of RA. GO analysis revealed the biological processes and the molecular functions that are highly regulated in RA by using the interaction of the merged protein. (Martin et al., 2010).

Molecular docking analysis was performed by the shortlisted compounds and their merged common targets which include TLR9, IDO1, TNF, KDR, JAK2, PTGS2, ALOX15, JAK1, ALOX5, TYK2. Analysis showed that only 2 compounds3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane) and 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide gave very high binding affinity to ALOX5, PTGS2, TYK2, and TNF.

Docking of ALOX5 with 3,3'-Isopropylidenebis(1,5,8,11-tetraoxacyclotridecane) scores -7.9. The functional domain of ALOX 5 is the PLAT domain having residues 2-118 and Lipoxygenase having residues 119-674. The binding residues to the ligands were present at these functional domains. Docking of TNF with 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide scores -7.2 and the functional domain of TNF is C-domain 1 having residues 22-833. Ligand docked with the residues of protein also lie in this domain. Docking of PTGS2 with 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide scores -7.9. The functional domain of the Peroxidase site having residues 97-598 and ligand docked with the residues of protein also lie in this domain. HIS (A:374) is a metal-binding site through which peroxidase activity was performed but the ligand binds at this site and blocks its activity and is predicted to be decreased in inflammation in RA. Docking of TYK2 with 2H-1,4-Benzothiazin-3(4H)-one, 4-hydroxy-2-methyl-, 1,1-dioxide scores -7.4. The functional domain of TYK2 is Protein Kinase domain 2 having residues 897-1176. The binding residues to the ligands were present at these functional domains. But ASN (A:1023) was the active site residue of the protein. Ligand docked at this site predicting to block its activation and resulting in a reduction in inflammation caused by arthritis.

Conclusion

Trachyspermum Ammi was used as herbal medicine because the anti-inflammatory and antioxidant activity of this plant proved its anti-arthritic nature. This plant has potential drug-like compounds that have these antioxidant and anti-inflammatory properties. Seven compounds were predicted to be druglike compounds out of which 2 compounds showed efficient binding to the targets.

Future Prospects

Future prospects of the study will be the in-silico validations of molecular docking results by Molecular Dynamic Simulation. Furthermore, Real-Time qPCR evaluation of potential predicted targets of *Trachyspermum Ammi* extracts and Compounds against Rheumatoid Arthritis.

Testing of *Trachyspermum Ammi* extracts and Compounds on in-vitro cell lines will also be done in near future to report the compounds of T. ammi plants a drug-like compounds.

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