Systematic Evaluation of Natural Compounds of *Thymus serpyllum* and *Cassia angustifolia* to Identify Potential Targets for Rheumatoid Arthritis

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2023

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A thesis submitted to National University of Sciences and Technology, Islamabad, in partial fulfillment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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2023

FORM TH-4

National University of Sciences & Technology

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Uzma Hafeez

Dedication

I dedicated this dissertation to all those who prayed for me and played their part in making me the person that I am today. May Allah bless you all!

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List of Abbreviations

IL-4	Interleukin-4
IL-6	Interleukin-6
JAK2	Janus Kinase 2
JAK-STAT	Janus Kinase and Signal Transducer And Activator of Transcription
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
RA	Rheumatoid Arthritis
STAT	Signal Transducer and Activator of Transcription
TNFαR	Tumor Necrosis Factor Alpha Receptor
Th1	T Helper 1 Cells
TIDM	Type 1 Diabetes Mellitus
TNF-α	Tumor Necrosis Factor Alpha
TYK2	Tyrosine Kinase 2

Abstract

Auto Immune are characterized by a wide variety of risk factors, which might include nutritional, environmental, and genetic variables. An autoimmune condition with a complicated origin is rheumatoid arthritis (RA). Rheumatoid Arthritis being treated majorly byNSAIDS, DMARDS, Glucocorticoids and Biologics, needs improvement in the therapy. Natural compounds from various plants have been used for centuries to cure various diseases owing to their fewer side effects. This study focuses on the natural compounds extracted from*Thymus serpyllum* and *Cassia Angustifolia* for their anti- rheumatic potential. The active phytochemicals of both the plants showed excellent characteristics to be used an anti-arthritic agent.as seen through DPPH Assay. The compounds from *T.serpyllum* and *C. Angustifolia* were screened for their potential targets against Rheumatoid Arthritis. The insilico docking analysis of phytochemicals and commercial drugs with potential targets of Rheumatoid Arthritis predicts a good binding energy to be a potential therapy substitute in comparison to the drugs available in the market with various side effects

Chapter 1

Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the most common chronic inflammatory joint disease and one of the diseases in which great progress has been made in the therapeutic aspects. RA mainly affects the mucous membranes of synovial joints and can cause progressive disability, premature death, and socioeconomic stress. (S. Jahangir, 2023). Clinical indicators of symmetrical inclusion of joints include arthralgia, swelling, redness, and limited range of motion. Initial diagnosis is considered an important improvement index for the most desirable outcomes, such as reduced joint demolition (van der Linden, 2010).

1.2 Epidemiology of RA

The cause of RA is unknown. The key word for this (and many other ways of tragedy) is that they are paid as a result of a person's exposure to environment or a trigger in genetically prone individual (Gibofsky, 1978).

1.3 Environmental Factors Role in Rheumatoid Arthritis

Other possible triggers for the environment are viral infections, such as Epstein-Barr virus and parvovirus, and bacterial infections with organisms such as Proteus and Mycoplasma.

Heat shock proteins and other stressors (e.g., changes in the hypothalamus-pituitary gland during adverse or traumatic life events) affect immune system regulation and cytokine production (McInnes IB, 2011).

Several environmental factors are capable of creating post-translational modifications of barrier tissues through peptidyl arginindemindemin type IV (PADI4), the enzyme responsible for post-translational citrullination of peptide antigens on arginine residues. PADI4 has the ability to modify citrullination of mucosal proteins and is associated with Porphyromonas gingivalis, which is present in periodontal disease and in patients who smoke cigarettes (McInnes IB, 2011). The following figure depicts the anomalies in the Ra patient joints with reference to the normal joints (Pradeepkiran, 2019).

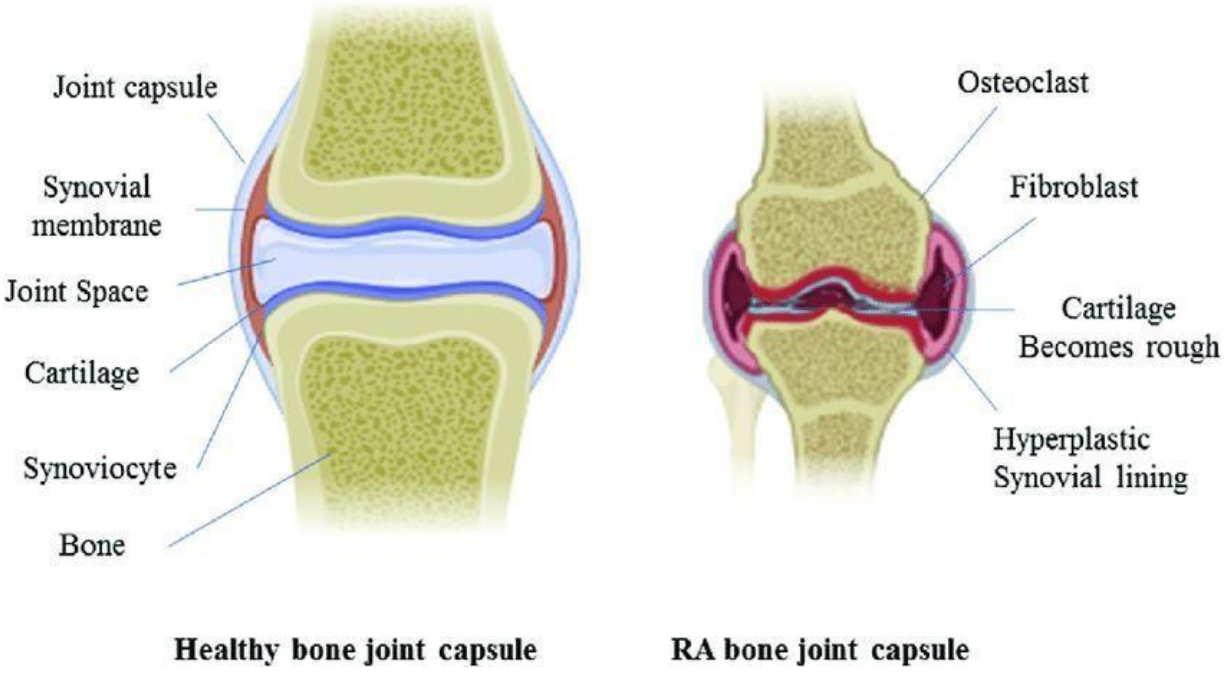


Figure 1 Bone joint in Rheumatoid Arthritis

1.4 Rheumatoid Arthritis linkage with other disorders

Mortality due to rheumatoid arthritis increases in chronic diseases, with one-third to half contributing only to cardiovascular disease. Nevertheless, infections, pneumonia, and, in severe cases, lymphoma also cause an increased mortality rate. Anemia, depression and osteoporosis are other less likely associated conditions (George E. Fragoulis, 2020).

Figure 2 Linkage of RA with other disorders

1.5 Role of fungus and bacteria in RA:

According to another hypothesis for RA pathophysiology, certain viral, bacterial and fungal strains as well as genetic and environmental factors play an important role in increasing the susceptibility of the disease (Victoria Ruffing, 2017).

The Th17 response is essential in inhibiting the colonization of Candida albicans, a fungus that increases the susceptibility to rheumatoid arthritis; however, Candida albicans infections have not been reported in many cases of the disease. However, it is assumed that several

inhibitors including TNF inhibitors and those blocking theTh17 response by inhibiting IL-17 and IL-23 play an important role in increasing the incidences of fungal and bacterial infections in rheumatoid arthritis patients (Shrinivas Bishu, 2014).

Porphyromonas gingivalis is one of the bacteria responsible for the onset of RA, ranging from gingivitis to periodontitis. It begins inflammation of gums, leading to collagen matrix and bone destruction (Chukkapalli S, 2016)**.** These citrullinated peptides are the major sponsors of immune cell activation and maydeteriorate the condition of rheumatoid arthritis (Białowąs K, 2014).

1.6 Factors Contributing in Rheumatoid Arthritis Development

There are certain factors that are involved in the development of Rheumatoid Arthritis.

1.61 Role of Genetics in Development of RA:

The onset of RA is reported by the activation of T cells, which in turn is activated by the binding of certain triggering peptides to T cell surface receptors. Human leukocyte antigen (HLA-DR4), a hyper-variable region of the major histocompatibility complex (MHC-II), is considered to be one of the highest genetic risk factors as it contributes to increasing the chances of RA by 30% (Victoria Ruffing, 2017). One of the other genetic factors is gene PAD4 that codes for an enzyme peptidyl arginine deiminase 4 (Mario Mellado, 2015).

This enzyme causes the citrullination of peptides leading to increased Plasma levels of anti-citrullinated peptide antibodies against citrullinated peptides ultimately leading to the onset of inflammatory pathways. CTLA44, PTPN22, and STAT4 are other important SNPs that are also found to play a role in RA (Júlia Kurkó, 2013).

1.62 Role of Cytokines in Development of RA:

Among the cytokines, the most prominent ones are TNF, IL-1 and IL-6. Others include IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-15, IL-17 and IL-23 (Mario Mellado L. M., 2015). IL-1, IL-6, and TNF are involved in inducing cytokine synthesis that upregulates adhesion molecules, such as E-selectin and intercellular adhesion molecule (ICAM) (Veale DJ, 1996).

Chapter 2

Literature Review

The musculoskeletal disorder rheumatoid arthritis (RA) affects the synovial joints and is systemic, polyarticular, chronic, progressive, and inflammatory.1 RA can also cause significant tissue damage to the heart2, as well as the lung, skin, eye, kidney, and blood vessels. Atypical innate, cellular, and humoral immunity define RA at the molecular and pathological levels.1,3-5 Therefore, abnormal proliferation kinetics leads to aberrant survival of activated T-lymphocytes, B-lymphocytes, mast cells, neutrophils, macrophages, accessory- antigen presenting cells (i.e., dendritic cells; DCs), synovial tissue fibroblasts7 (e.g., fibroblast-like synoviocytes), and accessory-antigen presenting cells (i.e., macrophages).

The typical single membrane synovium in RA synovial joints develops hyperplasia. This alteration is brought about by the enhanced adhesion and migration of activated immune and nonimmune cells. (S. Jahangir, 2023).

2.1 Inflammation in Rheumatoid Arthritis

Inflammation in the joints. IL-6 is also involved in activating B cells (Somaiya Mateen, 2016). IL-8 causes the recruitment of immune cells, IL-15 helps in the proliferation of T cells, IL-17 affects many cells, including osteoclasts, IL-23 is involved in Th17 cell differentiation, and GM-CSF plays a role in the development of macrophages. Activation ofall these cytokines leads to progression and deteriorating of disease condition by supportingand exacerbating inflammatory pathways (Bryl,2015).

2.2 Rheumatoid Arthritis Progression

Rheumatoid Arthritis Progression involves certain factors. These factors are discussed below:

2.21 T cell activation:

T cells are stimulated when they interact with mutated MHC molecules on the surface of antigen-presenting cells (APCs). However, T-cell activation entails additional co-stimulation via the CD28 receptor. If this co-stimulation does not transpire, T cells may endure apoptosis or are unable to be activated.

T cells-induced cytokines in return further activate T cells and other cells of the immune system. However, T cells may also directly stimulate the activation of immune cells by interacting with receptors present on the cell surfaces (Mario Mellado L. M.-M.-F., 2015).

2.22 B cell activation:

T cells interact with B cells to activate them and induce an immune response. B cells contribute by differentiating into plasma cells, interacting with other immune cells and cytokine production (Serena Bugatti, 2014).

2.23 Effector cell activation:

Activated B and T cells further activate effector cells, such as macrophages, which produce various pro-inflammatory cytokines. IL-1, IL-6, IL-8, TNF and GM-CSF are the prominent pro-inflammatory cytokines that have a role in activation and production of effector response in the micro-environment as well as in the distant parts of the body including liver through

various cell surface receptors (Alison Finnegan, 2012).

2.3 Treatment

NSAIDs, DMARDs and corticosteroids are the three medication approaches utilized for rheumatoid arthritis (Smolen J. S., 2016) (Victoria Ruffing, 2017)

The properly recognized DMARDs include:

Synthetic disease modifying anti-rheumatic drugs (sDMARD)

1. Methotrexate (MTX)
2. Leflunomide
3. Sulfasalazine

- Biological disease modifying anti-rheumatic drugs (bDMARDS)
- Tumor necrosis factor (TNF) inhibitors
    - Adalimumab
    - Certolizumab
    - Pegol
    - Etanercept
- Targeted synthetic (ts) DMARD
- Non-Steroidal Anti-Inflammatory Drugs
- Corticosteroids

These three kinds of drugs are used either as a monotherapy or in combination.

2.4 JAK Signaling pathways involved in Rheumatoid Arthritis

Signaling pathways play an important role in rheumatoid pathogenesis. Malfunctioning JAK/ pathway triggers rheumatoid arthritis. The JAK pathway is an important signaling mechanism involved in the induction of cytokine family interferons. JAK1, JAK2, JAK3, and TYK2 are non-receptor protein tyrosine kinases that belong to the Janus family of kinases (Malemud, 2013).

The inhibition of JAK activation has been shown to impact the activity of T-cells, natural killer cells, and dendritic cells, all of which play a crucial role in the progression and development of autoimmune diseases. Additionally, the effectiveness of JAK inhibition in preventing the downstream effects of type I/II cytokines has been demonstrated through pharmacological means (Malemud C. J., 2008).

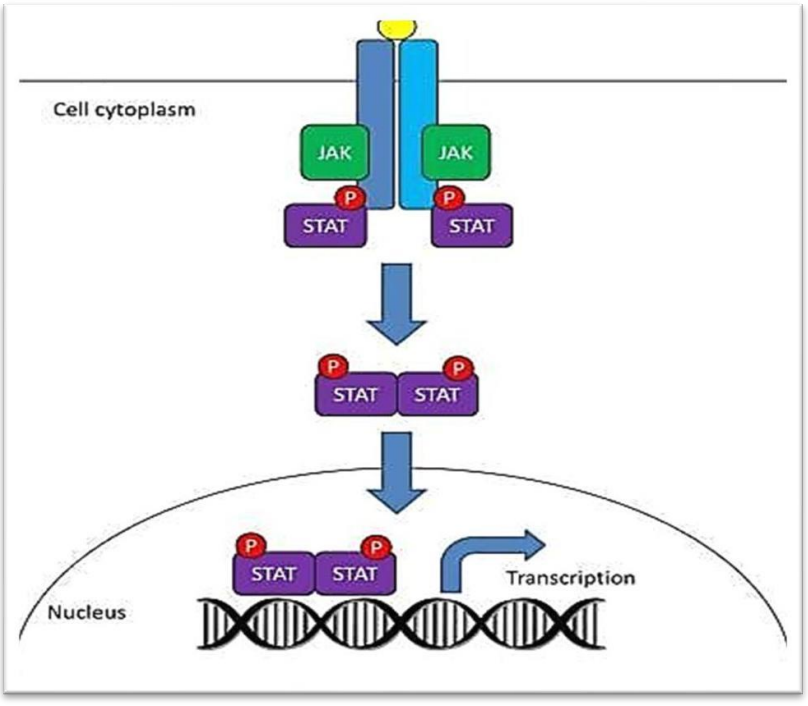


Figure 3 JAK STAT pathway in RA (Barnard, 2017)

2.5 Prostaglandins and Rheumatoid Arthritis

The early stages of rheumatoid arthritis (RA) cause the release of prostaglandins, which are lipid signaling molecules1. They can cause vasodilatation and are significant local inflammatory mediators. A diverse population of cellular and soluble immune system mediators, including T cells, B cells, macrophages, cytokines, and prostaglandins, infiltrate inflamed joints3. When stimulated, B cells can create prostaglandin E2 (PGE2), which helps to make RA4.

The phospholipase A2 (plpA2) family of enzymes releases arachidonic acid (AA) from the membrane phospholipids to produce prostaglandins, which are tiny, powerful inflammatory mediators. After that, cyclooxygenase (COX; prostaglandin endoperoxide H synthase; PGHS) and prostaglandin synthase enzymes convert AA into prostaglandins like PGE2, PGF2, PGD2, PGI2 (prostacyclin), and TXA2 (thromboxane), which are crucial for the regulation of physiological systems like the CNS and the responses to inflammation and the immune system [18, 19]. The two main isoforms of the cyclooxygenases, COX-1 and COX-2, are found in mammals and are heme-containing enzymes. Although COX-1 and COX-2 catalyze the same processes and share roughly 60% of the same amino acid sequence, they have distinct expression patterns and are encoded by different genes (Malemud C. J., 2009).

2.5 Importance of Herbal Medicines

When traditional treatment fails to work, use rises as a result of the growing drug resistance. The majority of people who live in impoverished nations get their healthcare mostlyfrom herbal medicines. Modern medications, dietary supplements, and food and beverage additives are being created using natural herbal items.

Because traditional medicines have beenused for thousands of years, there is a high degree of trust in their safetyand efficacy. Natural goods have become increasingly popular as a source of novel chemical compounds for the creation of contemporary pharmaceuticals as well as for usage as nutritional supplements, components of food and drink, phytocosmetics, and other herbal items (Chaughule, 2023).

2.6 Medicinal plants

In recent years, interest in ethno botanic, phytochemical and pharmacological research into the medicinal properties of T. Serpyllum, which serves as a highly efficient source for many different formulations in the pharmaceutical and chemical industries (S. Jaric, 2014).

Cassia Angustifolia, commonly called senna, is a well-growing balanced chemical used in Ayurvedic and modern drug preparations. C. Angustifolia leaves and pods were used for the treatment with anti-helminthic decoction powder. Angustifolia has been chiefly used an anti-pyretic in typhoid, splenic enlargement, cholera, anemia, toxicity, laxative, and genotoxicity stimulated by E. coli (S.I. Ahmed, 2016).

2.7 Insilco Drug Designing

In silico drug designing represents a cutting-edge approach in the field of pharmaceutical research, harnessing the power of computational methods to expedite the discovery of novel therapeutic compounds. This innovative technique involves the utilization of computer simulations and algorithms to predict the interactions between potential drug candidates and their target molecules within the human body.

By simulating these molecular interactions, researchers can rapidly screen thousands of compounds for their potential as drug candidates, significantly reducing the time and cost associated with traditional drug discovery methods.

Furthermore, in silico drug design allows for the customization of drug candidates, tailoring them to specific target proteins or pathways, thereby enhancing their efficacy and reducing potential side effects.

This approach not only expedites the drug development process but also holds great promise in addressing complex diseases for which traditional methods have fallen short. For more than three decades, computer-aided drug discovery (CADD) approaches have greatly benefited in the development of therapeutically relevant small molecules.

These methods are classified as structure-based or ligand-based. High-throughput screening and structure-based strategies are comparable in concept. Structure-based approaches include denovo ligand creation, and molecular docking

By combining the principles of biology, chemistry, and computational science, in silico drug designing is changing the pharmaceutical industry. Researchers can find compounds with high binding affinity and selectivity for their target proteins by utilizing cutting-edge algorithms and molecular modeling approaches, opening the door for the creation of more effective and precise medications. This technique lessens the need for costly materials and potentially hazardous substances during laboratory experiments. Despite the fact that in silico

drug design has many benefits, it is essential for researchers to regularly verify their computer predictions through actual experiments to guarantee the efficacy and safety of the produced medications (Athina Geronikaki, 2023).

Aims and Objectives

- To determine the phytochemicals of *Thymus serpyllum* and *Cassia angustifolia* and shortlisting of compounds.
- Identification of targets for the drug reposition of Rheumatoid Arthritis.

- Insilico evaluation of the shortlisted phytochemicals and commercially available drugs against Rheumatoid Arthritis through comparative molecular docking analysis and ligand protein interactions.

Chapter no 3

Methodology

3.1 Retrieval of Compound data of Thymus Serpyllum and CassiaAngustifolia

The compound data of *Thymus serpyllum* and *Cassia angustifolia* was collected from the following:

TCMSP- Traditional Chinese Medicine Systems Pharmacology

ChEMBL- Database of bioactivity data that links drug-like compounds to their biological targets

PubChem- Public Chemical Information Resource

Following parameters were collected of the compounds:

1. Molecular ID
2. Molecular weight
3. Hydrogen Donor
4. Hydrogen Acceptor
5. Oral Bioavailability
6. MLogP

3.11 Short listing of natural compounds of Thymus Serpyllum and Cassia Angustifolia

Out of all the collected compounds of both the plants, shortlisting was done by following Lipinski Rule, which says a compound is more likely to a drug if:

- ➔ Molecular mass less than 500 Dalton
- ➔ High lipophilicity (expressed as LogP less than 5)
- ➔ Less than 5 hydrogen bond donor
- ➔ Less than 10 hydrogen bond acceptors
- ➔ Molar refractivity should be between 40-130

In the next step all short-listed compounds were checked for **ADMET** properties which include as follows:

1. **Absorption**
2. **Distribution**
3. **Metabolism**
4. **Excretion**
5. **Toxicity**

These compounds SMILES were being run on ADMETSAR which is admetSAR which is a comprehensive source and tool for the prediction of chemical ADMET properties. Moreover, further shortlisting of natural compounds of both the compounds was done.

3.2 Retrieval of target data of Thymus serpyllum and Cassia Angustifolia

Targets for the compounds of *Thymus serpyllum* and *Cassia angustifolia* were collected using Swiss Target Prediction. The online application SwissTargetPrediction helps scientists forecast the targets of bioactivesmall compounds in humans and other animals. This is helpful to explain the molecular processes that give rise to a certain phenotypic or bioactivity, to anticipate off-target effects of known compounds, or to rationalize potential side effects (SwissTargetPrediction, 2013).

3.3 Rheumatoid Arthritis Targets Data Retrieval

About 280 targets for the Rheumatoid Arthritis were collected from literature andTherapeutic Target Database: TTD and TDR Targets

3.4 Retrieval of Commercial Drugs and their targets for RA

The commercial drugs that are being used in the market for the treatment of RA were being collected using the target data for Rheumatoid Arthritis as a reference (RheumatoidArthritis Treatment, 2020).

Table 1 Commercial Drugs for Rheumatoid Arthritis

S#	Commercial Drug Name
1	Rheumatrex
2	Azulfidine
3	Arava
4	adalimumab
5	abatacept
6	rituximab
7	azathioprine
8	infliximab
9	Leflunomide
10	Methotrexate
11	tofacinib

In the next step the targets of these specific drugs were scrutinized for the specific targets

3.5 Construction of Disease – Gene, Drug-Gene, and Compound - Targets Network Map

By using the software Cytoscape, the targets of Rheumatoid Arthritis were put through the STRING app to form a map.

In the same way the target data for rheumatoid arthritis was put through the STRING app to form a map.

3.6 Construction of Heterogeneous Network Cluster for short listed natural compounds

By merging all the short-listed data, a network cluster of drug-target-natural compounds, heterogeneous cluster was made depicting the

relationship between targets of Rheumatoid Arthritis, commercial drugs, and short-listed natural compounds of Thymus Serpyllum and Cassia Angustifolia.

3.7 Enrichment Analysis of target pathways of Rheumatoid Arthritis

Enrichment was performed on the merged targets from the cluster using ENRICHR software.

3.8 Docking of compounds, targets, and commercial drug

The Pdb of shortlisted compounds were taken from the NCBI database. Following steps were followed for Docking in MOE software.

- Pdb file of ligands and target were uploaded
- Polar hydrogens were added
- Energy minimization was performed
- Binding site allocation
- Docking

3.9 Preparation of Extracts and Maceration

In the next step, the samples were macerated with methanol and distilled water for three weeks. Four solutions in four different bottles were prepared for maceration:

A. Bottle 1: 50g of powdered leaves macerated with 500ml of methanol.

B. Bottle 2: 50g of powdered leaves macerated with 500ml of distilled water.

C. Bottle 3: 50g of powdered bark macerated with 500ml of methanol.

D. Bottle 4: 50g of powdered bark macerated with 500ml of distilled water.

Additionally, to prevent exposure to light, the bottles were wrapped with aluminum foil and placed in a dark room where they were shaken intermittently. The filtration process involved filtering the macerated bark and leaf solutions using Whatman filter paper to remove all insoluble components. The residue was discarded, and the filtrate was utilized for the subsequent steps of extract preparation.

3.10 Rotary evaporation

The purified filtrate obtained from the previous stage of filtration was further processed, where the excess solvent was removed using a rotary evaporator. The temperature of the water bath was adjusted to closely match the boiling point of the solvents, namely methanol and distilled water. After an hour of evaporation at 74°C of the methanolic filtrates, a sticky methanolic extract of leaves and a methanolic extract of bark were obtained. However, for the aqueous extracts, the filtrate was subjected to more than four hours of rotary evaporation at 98°C to evaporate the water and obtain a sticky aqueous extract of leaves and a powdered aqueous extract.

3.11 Phytochemical Tests

Phytochemicals were identified through phytochemical analysis, which involved the use of methanol and distilled water as solvents for preparing extracts. The tests were conducted in duplicates, with controls being equated to the sample extracts. To begin, reagents were prepared for various tests, and three tubes were used to perform one control reaction with distilled water and the other two for sample extracts. Various indicators, such as color changes, precipitate formation, foam appearance, and organic layer formation, were used to confirm the presence of phytochemicals. Details of the reagents and tests performed are as follows:

A. Alkaloids: Hager's Test

- Reagent: Hager's reagent (1g Picric Acid in 100 ml of distilled water)
- Procedure: 250 µl extract + few drops of Hager's reagent
- Final observation: yellow precipitate

B. Phenols

- Procedure: 250 µl extract + few drops of 1% FeCl3 (For 1% FeCl3: 1 g of FeCl3 in 100 ml of distilled water)
- Final observation: Bluish Black color

C. Terpenoids

- Procedure: 250 µl extract + 250 µl Ethanol + 250 µl CHCl3 + Heat (2 min) + few drops ofconcentrated H2SO4
- Final observation: red violet color

D. Flavonoids

- Procedure: 250 µl extract + 250 µl 10% lead acetate
- Final observation: yellow precipitate

3.12 DPPH Assay

DPPH assay was performed using 2,2-diphenyl-1-picrylhydrazyl frequently known as DPPH. This organic compound is a rich supplier of stable free radicals and is used to examine the in-vitro antioxidant activity of our samples. DPPH, a crystalline compound when disbanded in solvent yields a purple-colored solution that is photo-sensitive and is arranged just before performing the assay (Singh, 2011).

Figure 4 DPPH reaction mixture being prepared at 96 well microplate

A. DPPH Protocol:

For performing the test, equal concentrations of DPPH and test samples were mixed, shaken, and incubated in dark for half an hour. In this analysis, the test samples are the aqueous and methanolic extracts of both T. Serpyllum and C. Angustifolia.Different dilutions of test samples were prepared and assessed against dilutions of a standard.Ascorbic acid was used as a standard or positive control to compare the % inhibition of our samples. To perform the assay, sample dilutions, positive control dilutions, negative controls and DPPH solution were

made.

B. Sample dilutions

Sample dilutions of methanolic and aqueous extracts were taken in following decreasing concentrations were prepared:

- Dilution 1: 140µl/ml
- Dilution 2: 120µl/ml
- Dilution 3: 100µl/ml
- Dilution 4: 80µl/ml
- Dilution 5: 60µl/ml
- Dilution 6: 40µl/ml

C. Positive control (Ascorbic acid)Dilutions

Control dilutions in following decreasing concentrations were prepared:

Dilution 1: 140µl/ml

- Dilution 2: 120µl/
- Dilution 3: 100µl/ml
- Dilution 4: 80µl/ml
- Dilution 5: 60µl/ml
- Dilution 6: 40µl/ml

D. Negative controls

DPPH + methanol

E. Negative control 2:

DPPH + distilled water

0.1mM DPPH was prepared by dissolving 3.94mg of DPPH in 100ml of methanol. The flask

was covered with aluminum foil to protect the solution from light and was shaken meticulously to dissolve the DPPH in methanol.

All the dilutions of test samples and the control were made in triplicates. 500µl of eachdilution was mixed with 500µl of DPPH solution, shook appropriately and then was incubated in dark for half an hour for the reaction to take place. (Singh, 2011)

Two negative controls were made, one having 500µl of DPPH in 500µl of distilled water and the other having 500µl of DPPH in 500µl of methanol were also arranged and incubated in dark for half an hour. Optical density was evaluated utilizing the UV VIS spectrophotometer.

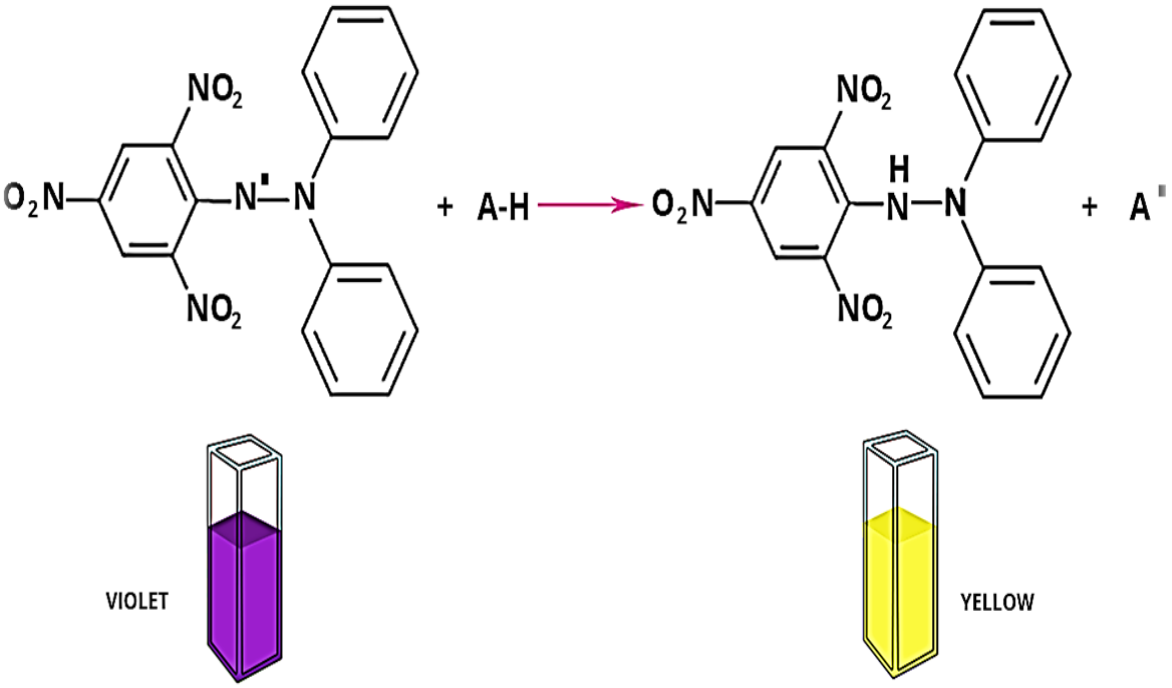


Figure 5 Determination of the activity of an antioxidant by the DPPH assay (Maillard, 2015).

3.13 Albumin Denaturation Assay

The reaction mixtures were heated in a water bath to 70°C and kept there for 5 minutes after being incubated at 37°C 2°C for 15-20 min. The reaction mixture was then given 15 minutes to cool at room temperature. A colorimeter was used to test the reaction mixture's absorbance at each concentration before and after denaturation. Three times each test was run, and the mean absorbance was noted. The following formula was used to calculate the percentage of protein inhibition in relation to the control.

Chapter no 4

Results

4.1 Shortlisted compounds of Thymus Serpyllum and Cassia Angustifolia

Following is the list of Thymus Serpyllum and Cassia Angustifolia compounds thatwere shortlisted.

Table 2 Shortlisted compounds of Thymus Serpyllum

Compound	MLOGP	Hydrogen Donor	Hydrogen Acceptor	OraB%
Borneol	3.86	1	1	81.8
Thymol	2.95	1	1	41.47
Carvacrol	4.63	1	1	43.28

Table 3 Shortlisted compounds of Cassia Angustifolia

Compound	MLOG P	Hydrogen Donor	Hydrogen Acceptor	OB%
Benzeneacetic acid, alpha-[(tert-butyldimethylsilyl)oxy]-, tert-butyldimethylsilyl ester	3.96	0	3	25.94
1-(3-(Cyclohexylamino) propyl) guanidine	2.45	3	2	51.89

4.2 Cytoscape Networking

To visualize molecular interaction networks and integrate them with gene expression profiles and other statedata, use the free and open-source bioinformatics software platform known as Cytoscape. Plugins that add further functions are available. There are plugins for large-scale network searching, molecular profiling analyses, new layouts, additional file format support, and connectivity with databases.The network of all RAtargets was merged on cytoscape through STRING app is shown in the figure 4:

In this figure which was generated from string database, all the disconnected nodes were deleted from the network. The contact is shown by the line, while the sphere represents the node (the protein expressed by the gene). (dark green), empirically determined (magenta), and curated databases (blue). The internalsphere structure is a representation of the protein's 3D structure

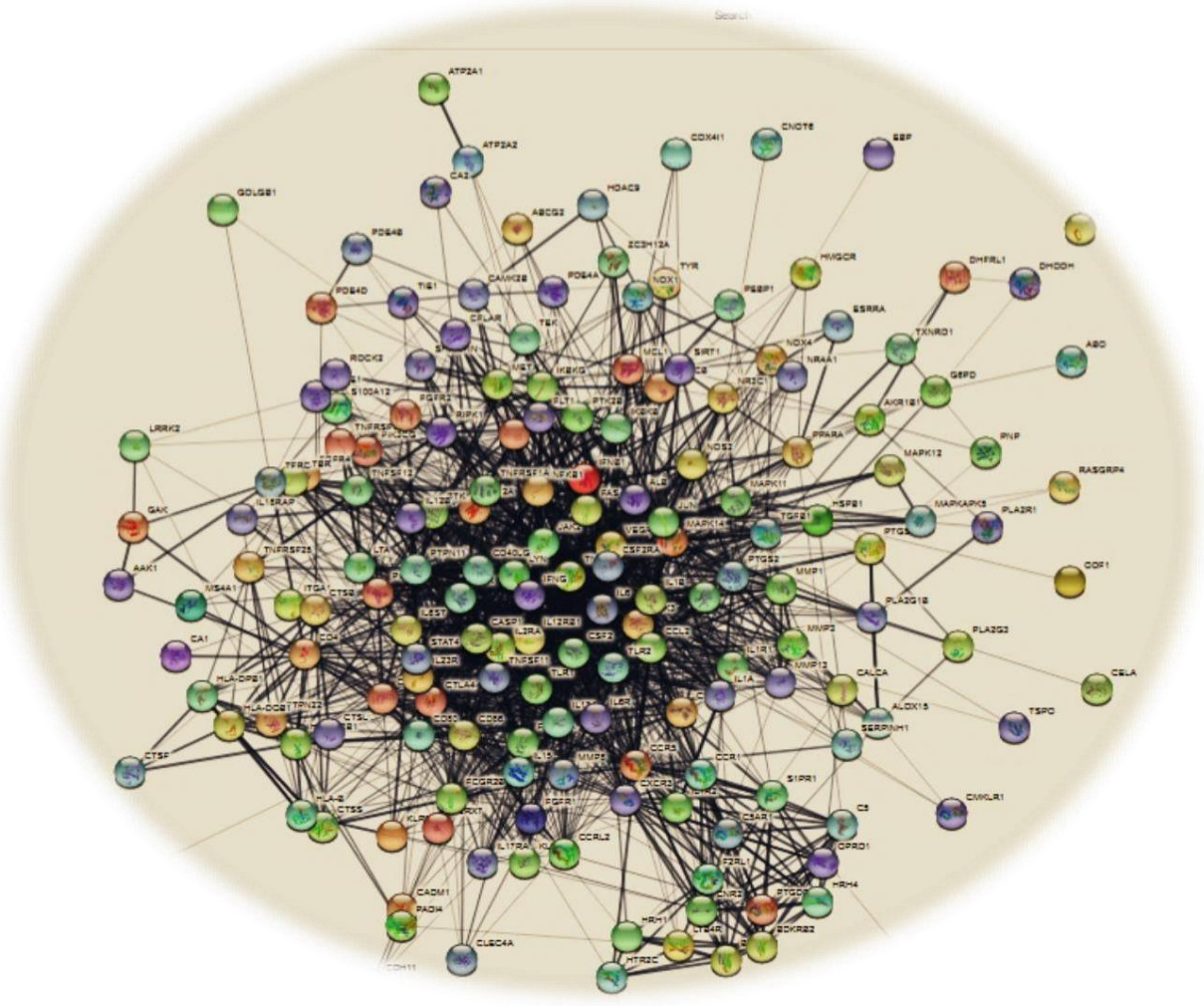


Figure 6 Rheumatoid Arthritis Targets Network developed from cytoscape

4.3 Common targets of Thymus Serpyllum and RA

Similarly, the targets of Thymus Serpyllum and Cassia Angustifolia were run on cytoscape to get a network. The networks of both of these plants were then individually merged to retrieve the common targets for Rheumatoid arthritis.

Table 4 Common targets of Thymus Serpyllum and RA

Target Symbol	Target name
PTGS2	Cytochrome c oxidase subunit 2
NR3C1	Glucocorticoid receptor
DHODH	Dihydroorotate dehydrogenase (quinone)
HTR2C	5-hydroxytryptamine receptor-2C
JAK2	Tyrosine-protein kinase JAK2
OPRD1	Opioid receptor
NR4A1	Nuclear receptor subfamily 4
CA1	Carbonic anhydrase 2
G6PD	Glucose-6-phosphate 1-dehydrogenase
P2RX7	P2X purinoceptor 7
MMP13	Matrix metallopeptidase 13
P2RX7	P2X purinoceptor 7

Target Symbol	Target name
PTGS1	Cyclooxygenase 1
JAK2	Tyrosine-protein kinase JAK2
CA2	Carbonic anhydrase 2
HRH4	Histamine h4 receptor
CTSL	Cathepsin L
HTR2C	5-hydroxytryptamine receptor-2C
JAK3	Janus kinase3
NOS2	Nitric oxide synthase
BACE1	Beta-secretase1
PTGS1	Cyclooxygenase 1
JAK2	Tyrosine-protein kinase JAK2
TYR	tyrosine

4.4 Common targets of Cassia Angustifolia and Rheumatoid Arthritis

The common targets of Cassia Angustifolia and Rheumatoid Arthritis retrieved are shown the table as follows:

Table 5 Common targets of Cassia Angustifolia and RA

Target Symbol	Target name
CA2	Carbonic anhydrase 2
PTGS2	Cycloxygenase2
CA1	Carbonic anhydrase 1
CTSS	Cathepsin s
PPARA	Peroxisome proliferator-activated receptor
OPRD1	Opioid receptor
LRRK2	Leucine repeats rich kinase
P2RX7	Purinergic receptor
BACE1	Beta- secretase 1
DHODH	Dihydroorotate dehydrogenase (quinone)
G6PD	Glucose-6-phosphate 1-dehydrogenase
NOS2	Nitric oxide synthase
MAPK14	Mitogen-activated protein kinase

4.5 Cytoscape network of common targets of RA and compounds from *T.Serpyllum* and *C. Angustifolia*

The following network map was generated of each plant with the disease i.e. Rheumatoid Arthritis to identify the potential target genes and to analyze the complex networks of the targets. It was generated by merging all the networks of each phytochemical by selecting the intersection option in the interface.

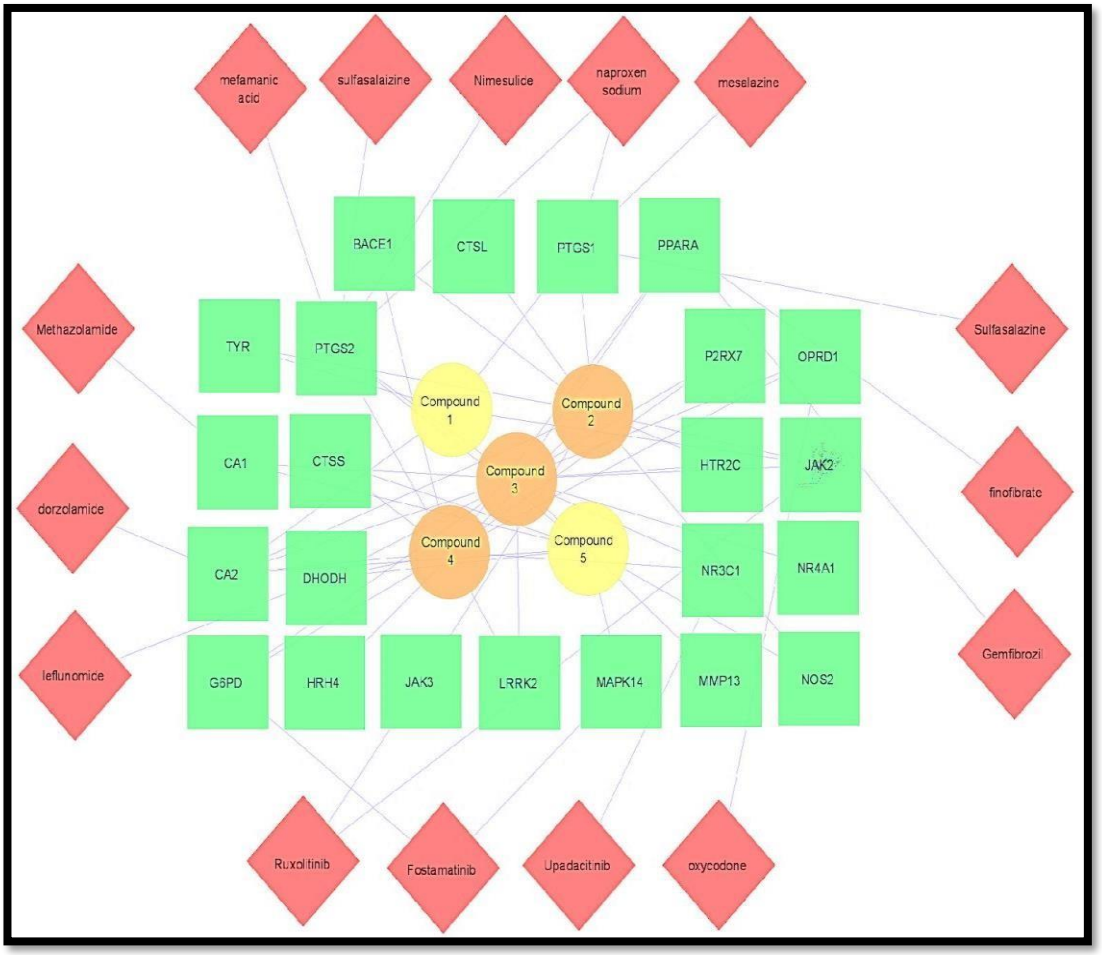


Figure 7 Cytoscape Network of RA targets with compounds

4.6 Phytochemical screening results

The results for the phytochemical screening showed that both the plants showedthe presence of Alkaloids, Phenols, Terpenoids and Flavonoids.

Table 6 Phytochemical screening Results

Test name	**T.serpyllum results**	**C.Angustifolia Results**
Alkaloids	++	++
Phenols	++	++
Terpenoids	++	++
Flavonoids	++	++

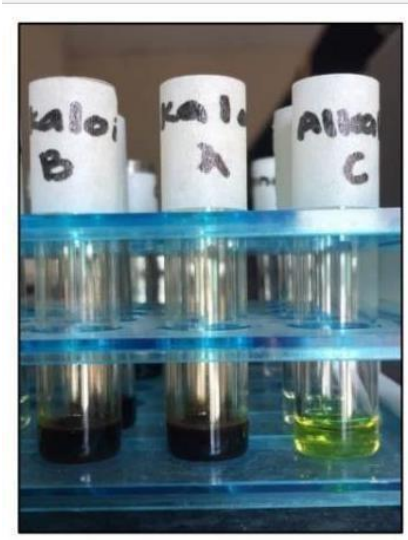


Figure 8 Alkaloids Test

Figure 9 Terpenoids Test

Figure 10 Anthraquinones Test

Figure 11 Phenols Test

4.7 Results of DPPH Assay

Results of DPPH assay were assessed by measuring % inhibition of the extractsusing the following formula:

% inhibition = (Absorbance of Control - Absorbance of Sample/ Absorbance ofControl) *100

Where, Control = DPPH + Solvent Sample = DPPH + Extract

The extracts have shown a significant rise in scavenging the free DPPH radicalspresent in the solution.

The following graph shows the increasing trend of % inhibition with increasingconcentration of the sample extracts and is compared with the positive controlof ascorbic acid.

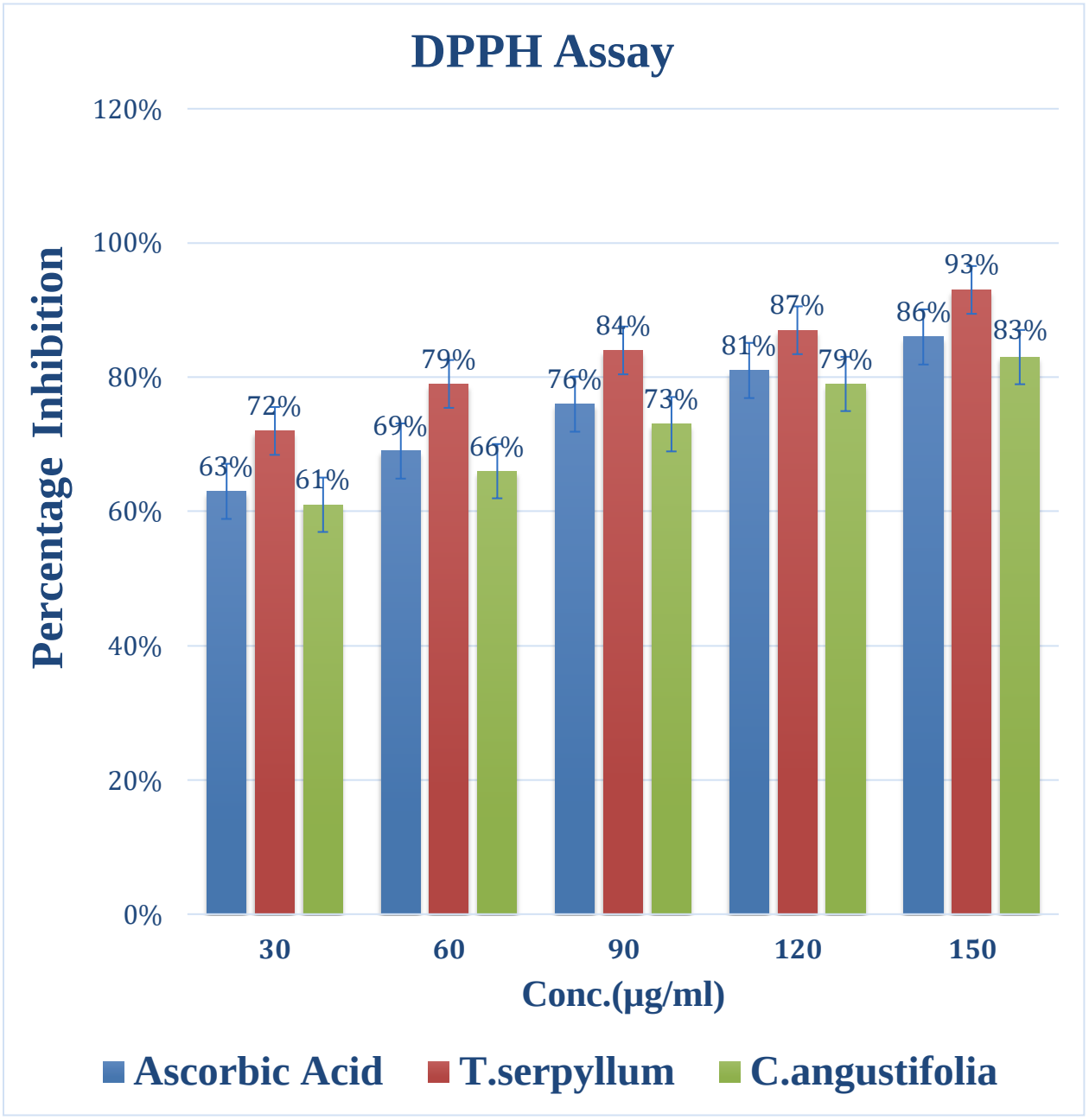


Figure 12 Graphical Representation of DPPH Results of T.Serpyllum and C. Angutifolia

The graph depicts that the percentage inhibition of the compounds of Thymus Serphylum and Cassia Angustifolia shows increase with the increase in the concentrations of compounds.

4.8 Results of Albumin Denaturation Assay

Results of Albumin Denaturation Assay were assessed by measuring %inhibition of the extracts using the following formula:

Percentage inhibition (%) =

$$\left(\frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

The following graph shows the increasing trend of % inhibition with increasingconcentration of the sample extracts and is compared with the positive controlibuprofen.

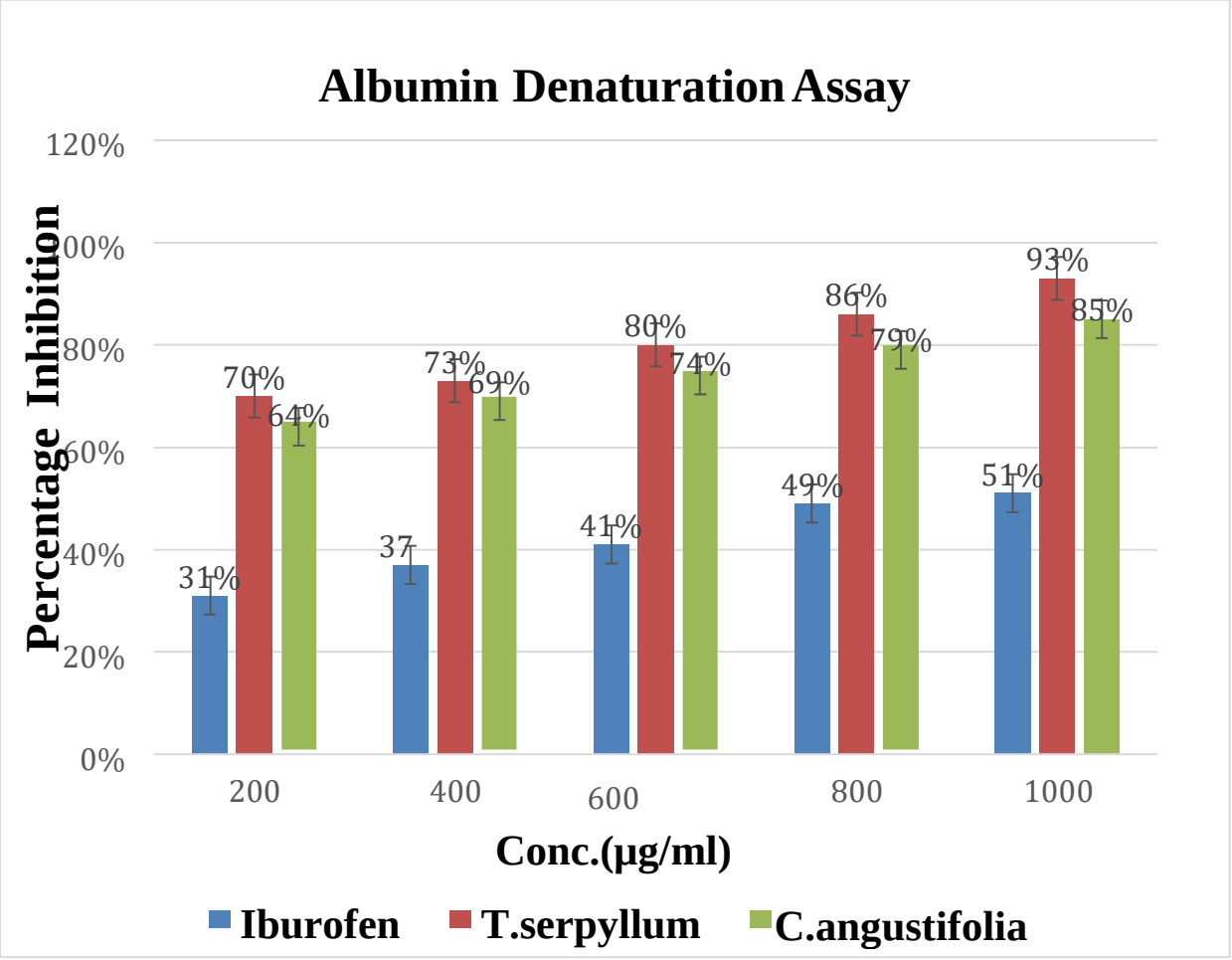
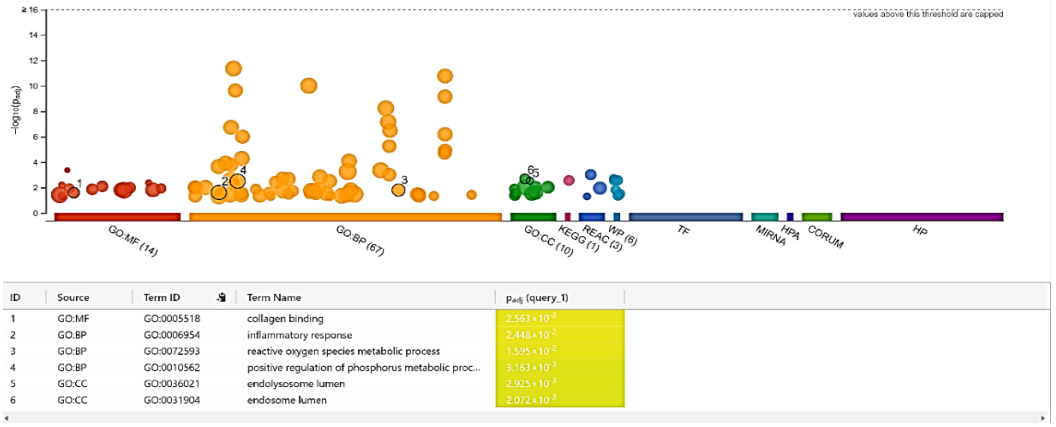


Figure 13 Graphical Representation of Albumin Denaturation Results

4.8 Enrichment Analysis

Following are the results of the enrichment analysis performed at Enrichr Software. The label 1 shows the receptors for collagen binding, label 2 shows the receptors for inflammatory response, label 3 for ROS (reactive Oxygen Species) Metabolic Process and 4 for positive regulation of phosphorus metabolic process, respectively.



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0005518	collagen binding	2.563×10^{-2}
2	GO:BP	GO:0006954	inflammatory response	2.448×10^{-2}
3	GO:BP	GO:0072593	reactive oxygen species metabolic process	1.595×10^{-2}
4	GO:BP	GO:0010562	positive regulation of phosphorus metabolic proc...	3.163×10^{-3}
5	GO:CC	GO:0036021	endolysosome lumen	2.925×10^{-3}
6	GO:CC	GO:0031904	endosome lumen	2.072×10^{-3}

Figure 14 Graphical Representation of Enrichment Analysis from Enrichr Software

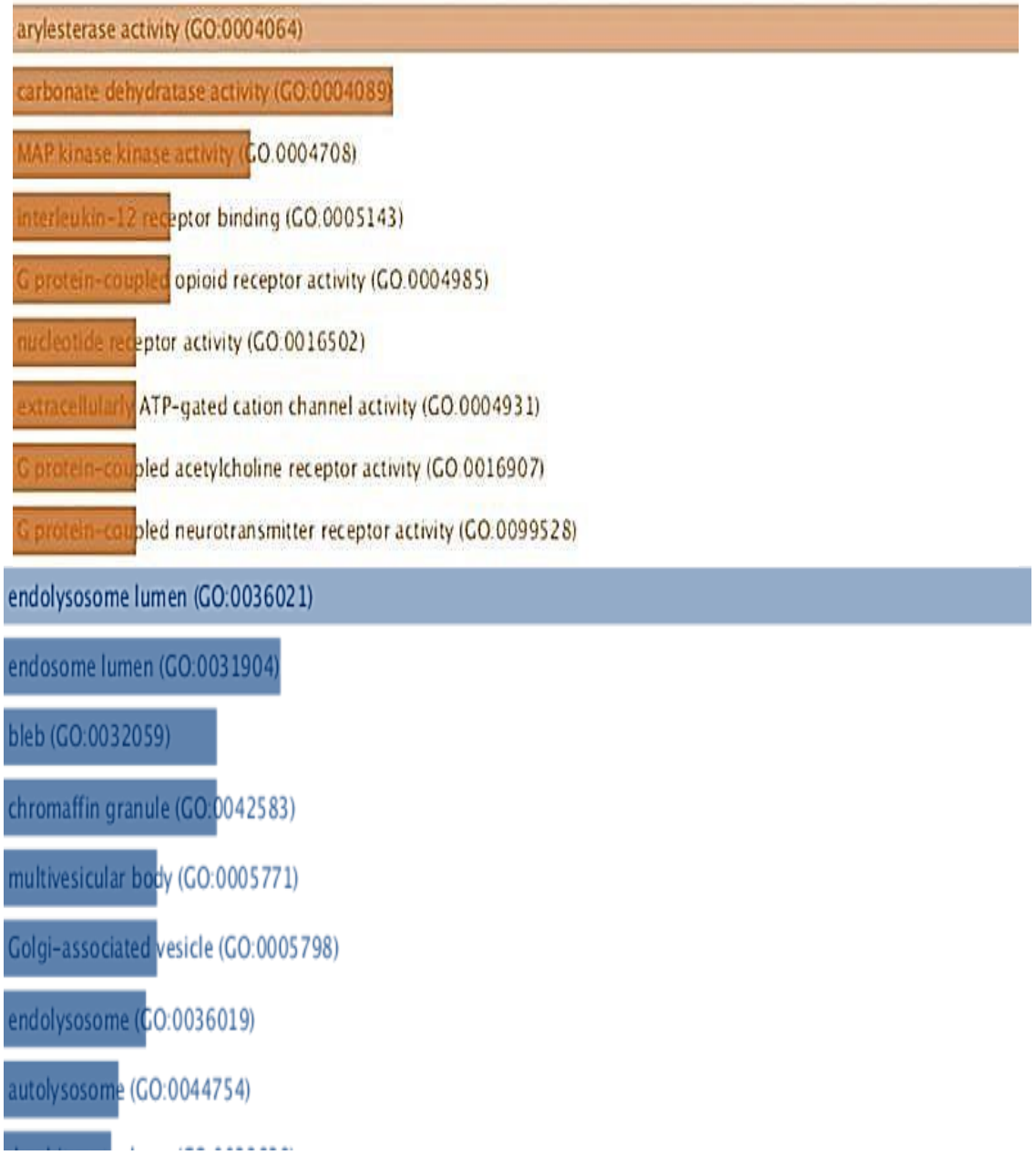


Figure 15 Enrichment Analysis (from top) of Biological Processes, Cellular Compartments and molecular functions

Enrichment Analysis showed the highest presence of tyrosine phosphorylation of STAT protein in biological processes enrichment, for Cellular Compartments, endosome lumen has the highest presence and for molecular functions arylesterase activity has the highest presence.

4.9 Molecular Docking

Top compounds that were shortlisted in the previous steps were then used for the molecular docking analysis through MOE software.

For docking study specifically JAK-Kinase were taken as target proteins.Three Dimensional Structures of target proteins were available on Protein Data Bank (PDB) therefore, they were downloaded in PDB format.

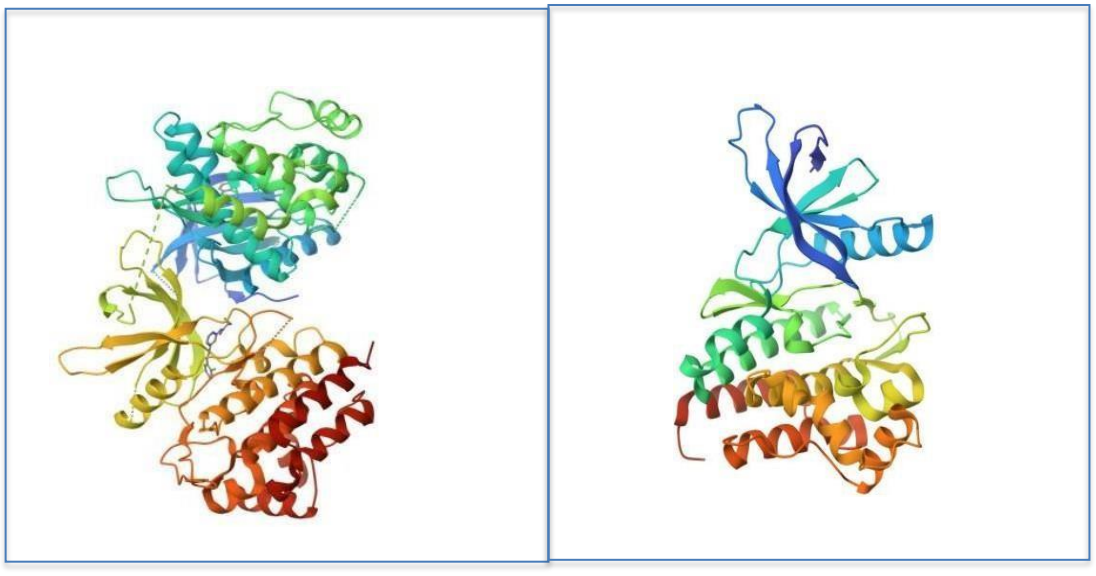


Figure 16 3D structure of Jaunus Kinase JAK 3(left) and Janus Kinase 2 as retrieved from Protein Data Base

4.91 JAK2 Docking Results with Ruxolitinib

The docking of the commercial drug Ruxolitinib with JAK 2 target protein showed the following results

Table 7 Result of docking of JAK2 with Ruxolitinib

S#	mol	mseq	S	rmsd-refine	E-Conf	E-place	E-score	E-refine	E-score2
1	**ruxolitinib**	**1**	**-11.4**	**2.16**	**7.06**	**-63.07**	**-10.57**	**-0.63**	**-11.43**

The Root Mean Square Deviation (rmsd) value of commercial drug is 2.16 when docked withJAK 2 target. The ligand interactions as derived from MOE software are as follows

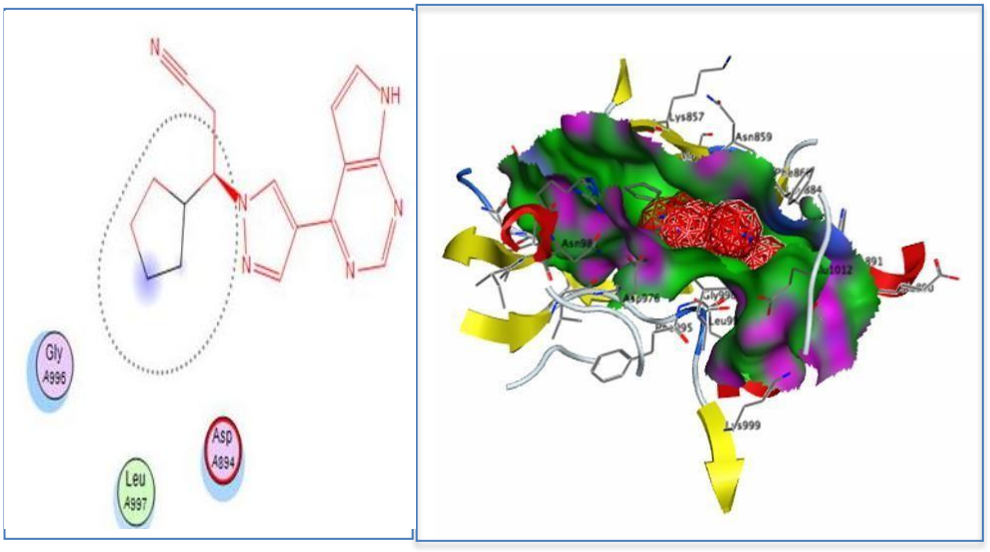


Figure 17 2-Dimensional interaction of JAK2 (left) and 3- Dimensional interaction of JAK2with Ruxolitinib from MOE software

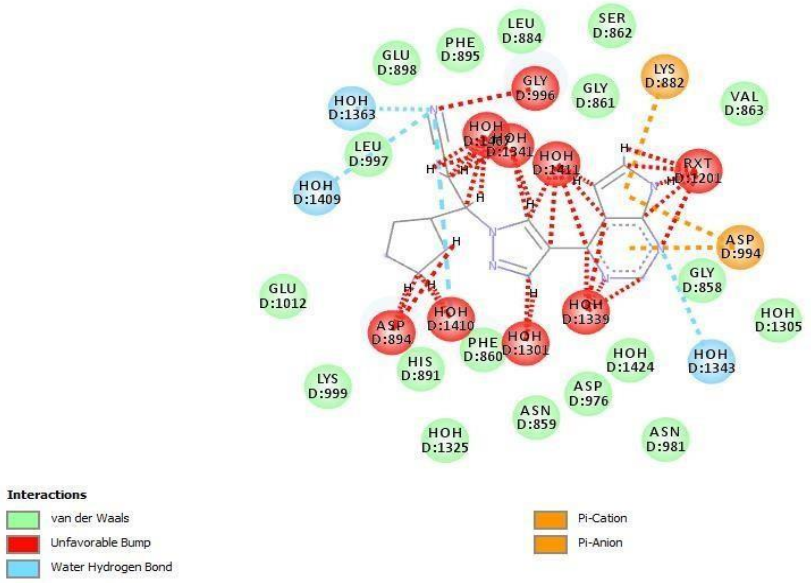


Figure 18 2-Dimensional interaction of JAK2 with Ruxolitinib from Biovia

4.92 JAK 2 Docking Results of with Borneol

The docking of the phytochemical Borneol with JAK 2 target protein showed the following results

Table 8 Result of docking of JAK2 with Borneol

S#	mol	mseq	S	rmsd-refine	E-Conf	E-place	E-score	E-refine	E-score2
1	**borneol**	**2**	**-8.59**	**0.4468**	**59.95**	**53.14**	**-8.10**	**11.58**	**-8.59**

The rmsd value of the docking results of borneol with jak 2 is 0.44 which is significantly a more desirable value in comparison to the commercial drug. The ligand interactions as derived from MOE software are as follows

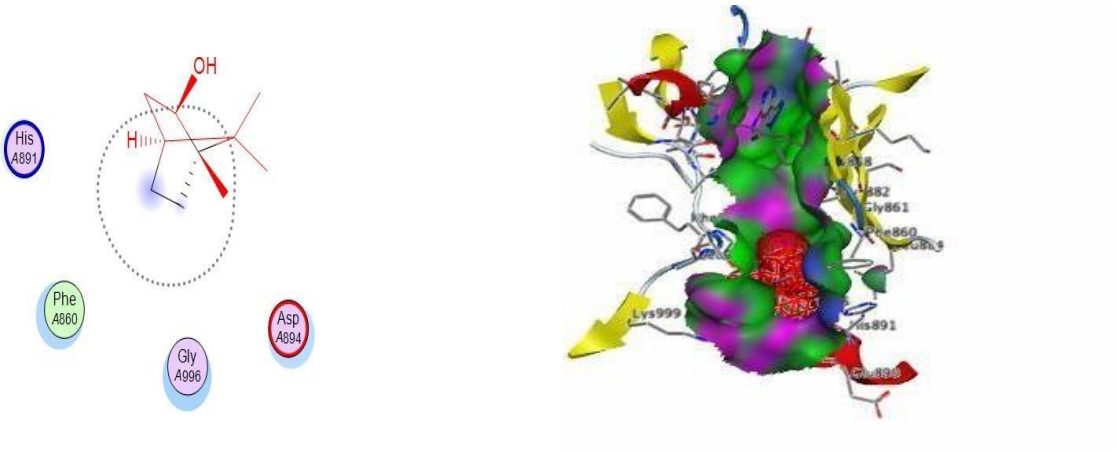


Figure 19 2-Dimensional interaction of docking of JAK2 with Borneol (left)and 3-Dimensional interaction of docking of JAK2 with Borneol from MOE

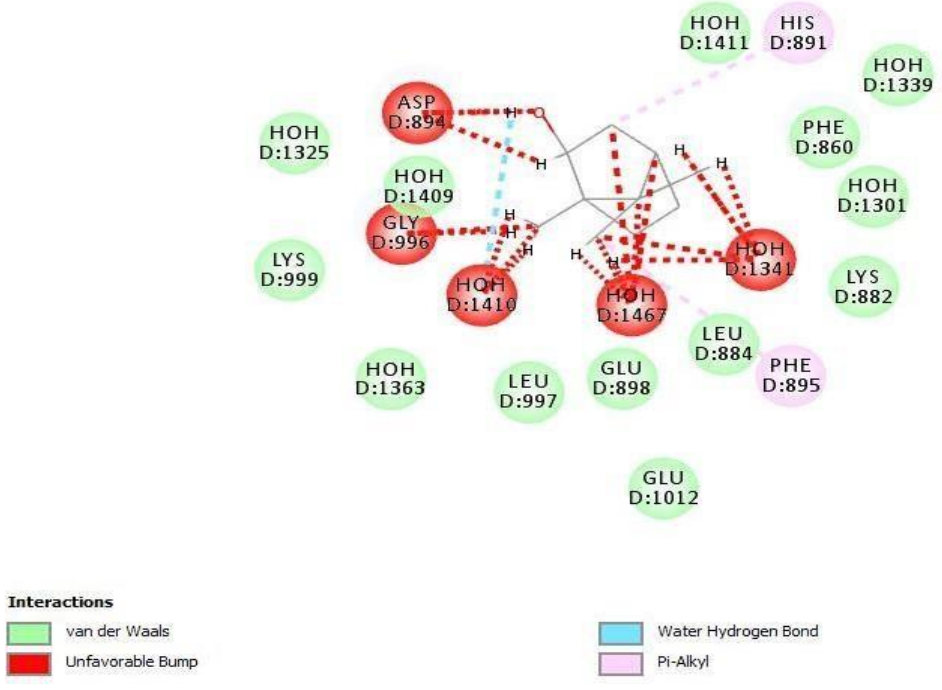


Figure 20 2-Dimensional interaction of docking of JAK2 with Borneol from Biovia

4.93 Docking Results of JAK 2 With Thymol:

Docking of the phytochemical Thymol with JAK 2 target protein showed the following results

Table 9Result of docking of JAK2 with Thymol

S#	mol	mseq	S	rmsd-refine	E-Conf	E-place	E-score	E-refine	E-score2
1	**thymol**	**4**	**-10.16**	**0.59**	**24.35**	**49.65**	**-9.04**	**11.8820**	**-10.16**

The rmsd value of the docking results of Thymol with jak 2 is 0.59 which is significantly a more desirable value in comparison to the commercial drug. The ligand interactions as derived from MOE software are as follows

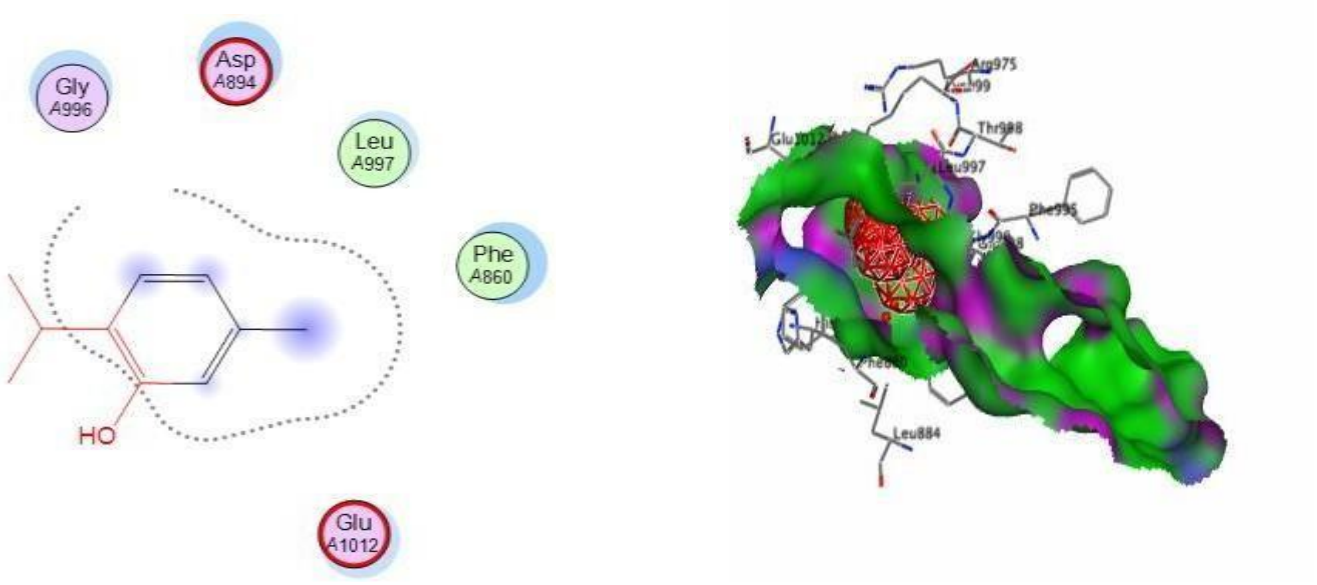


Figure 21 2-Dimensional Result of docking of JAK2 with Thymol(left) and 3-Dimensional Result of docking of JAK2 with Thymol from MOE software

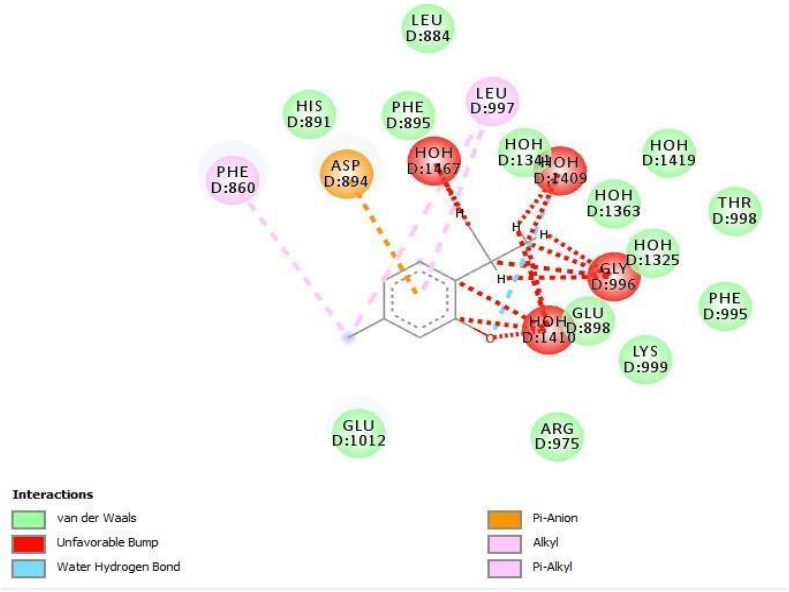


Figure 22 2-Dimensional Result of interaction of JAK2 with Thymol from Biovia

4.94 Docking Results of JAK 2 with carvaceol

Docking of the phytochemical carvaceol with JAK 2 target protein showed the following results

Table 10 Result of docking of JAK2 with carvacrol

S#	mol	mseq	S	rmsd-refine	E-Conf	E-place	E-score	E-refine	E-score2
1	**Carvaceol**	**3**	**-9.6**	**0.98**	**11.35**	**52.15**	**-9.86**	**-6.77**	**-9.61**

The rmsd value of the docking results of carvaceol with jak 2 is 0.98 which is significantly a more desirable value in comparison to the commercial drug. The ligand interactions as derived from MOE software are as follows

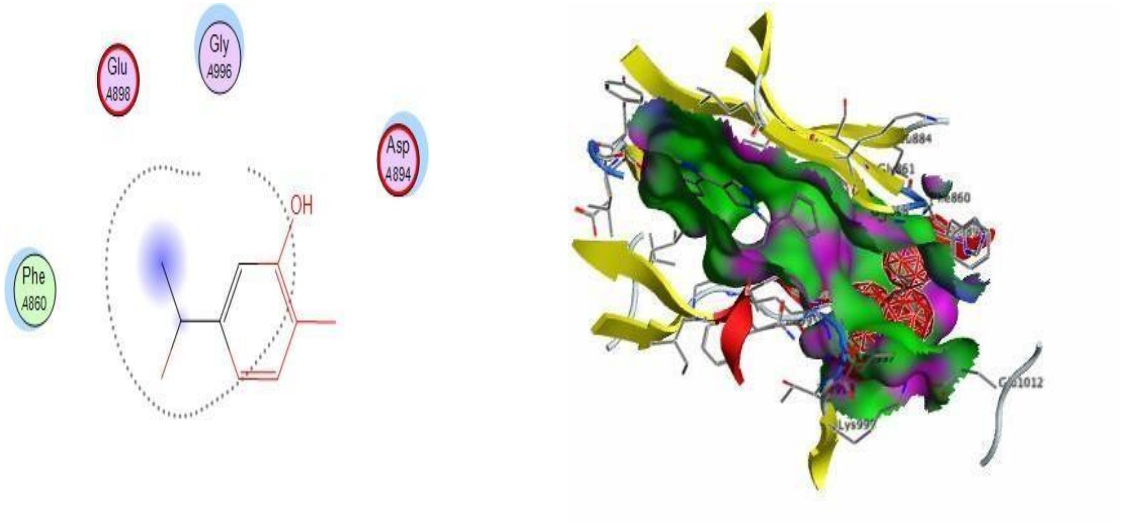


Figure 23 2-Dimensional Result of docking of JAK2 with carvaceol (left) and 3--Dimensional Result of docking of JAK2 with carvaceol from MOE

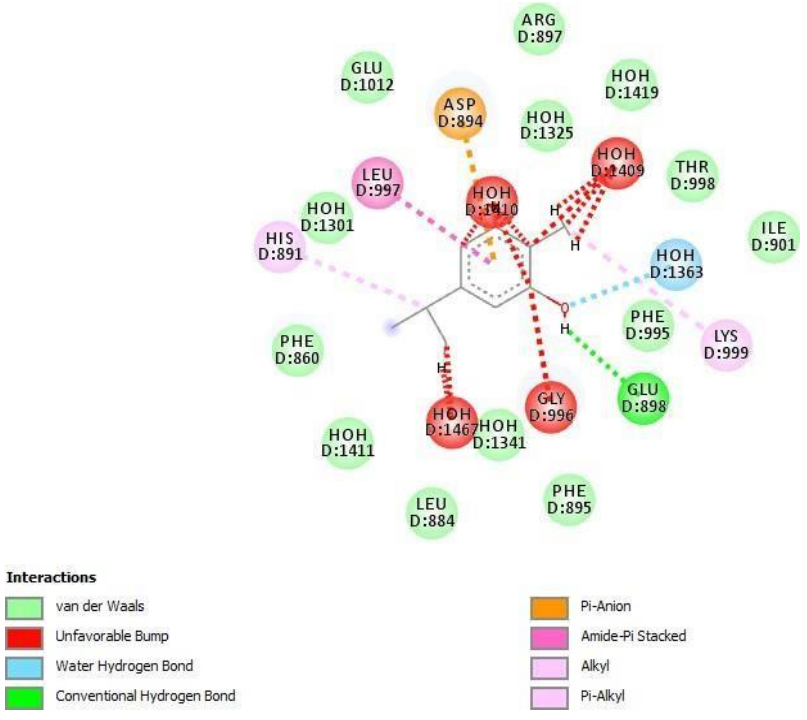


Figure 24 2-Dimensional interactions of JAK2 with Thymol from Biovia

4.95 Docking Results of JAK3 with Thymol

Docking of the phytochemical thymol with JAK 3 target protein showed the following results

Table 11 Result of docking of JAK3 with Thymol

S#	mol	mseq	S	rmsd-refine	E-Conf	E-plaace	e-score	E-refine	E-score2
1	**thymol**	**2**	**-9.90**	**1.29**	**10.78**	**–39.96**	**-10.80**	**-7.71**	**-9.90**

The rmsd value of the docking results of thymol with jak 3 is 1.29 which is significantly a more desirable value in comparison to the commercial drug. The ligand interactions as derived from MOE software are as follows

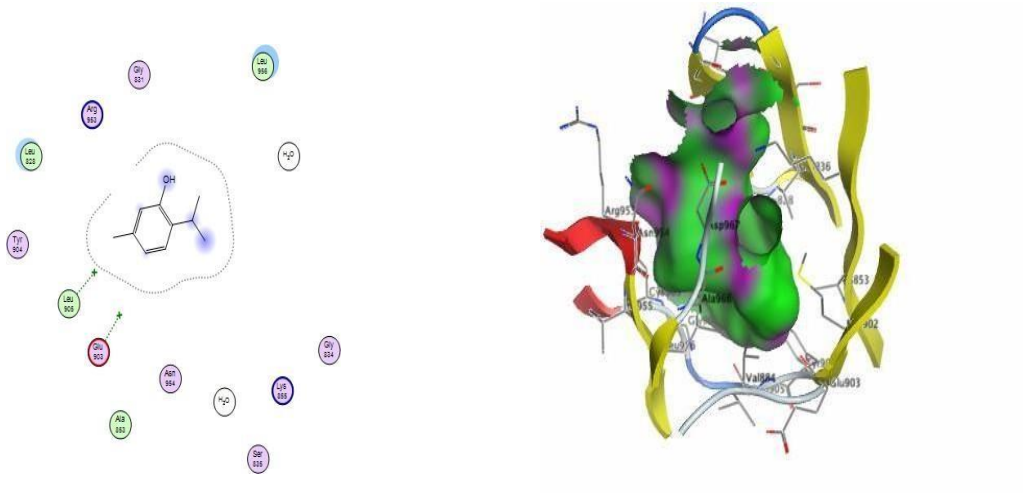


Figure 25 2-Dimensional Result of docking of JAK3 with Thymol(left)and 3-Dimensional Result of docking of JAK3 with Thymol from MOE

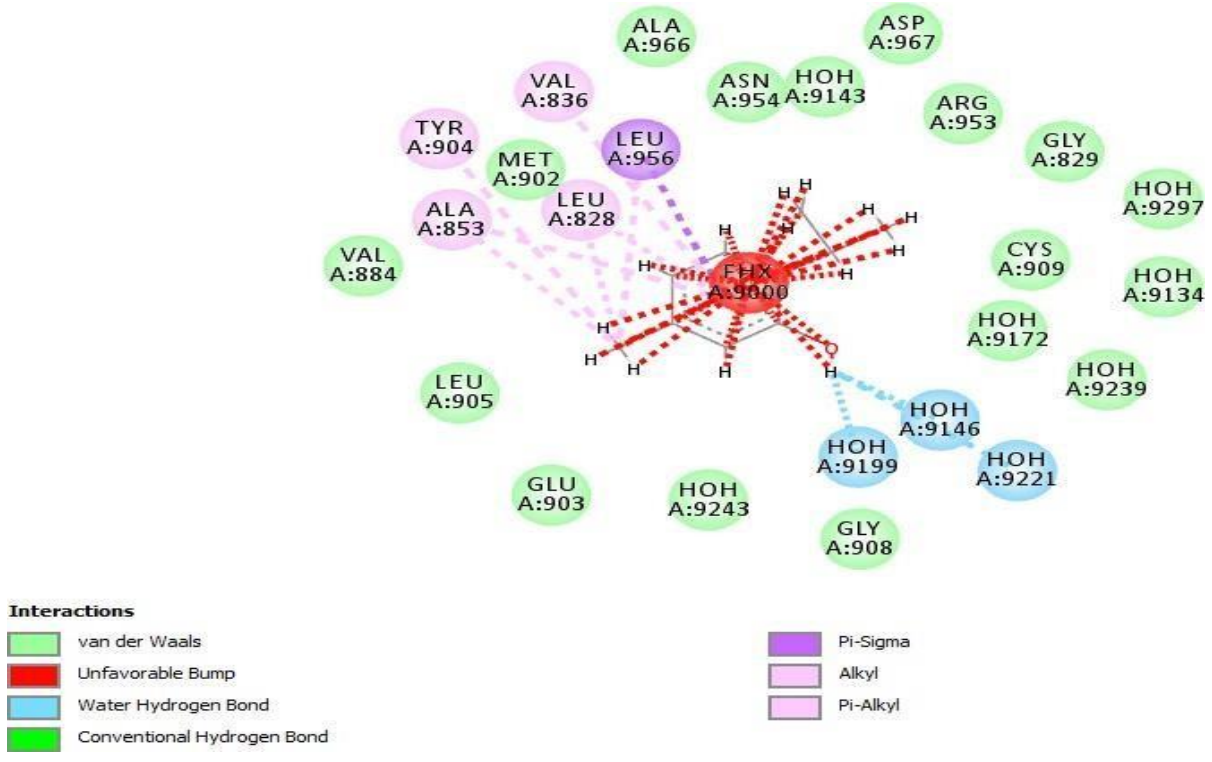


Figure 26 2-Dimensional Interactions of JAK3 with Thymol from Biovia

4.96 Docking Results of JAK3 with Ruxolitinib:

Docking of the commercial drug with JAK 3 target protein showed the following results

Table 12Result of docking of JAK3 with Ruxolitinib

S#	mol	mseq	S	rmsd-refine	E-Conf	E-place	e-score	E-refine	E-score2
1	**ruxolitinib**	**1**	**-8.77**	**2.42**	**7.23**	**-31.01**	**-10.39**	**-9.94**	**-8.77**

The rmsd value of the docking results of the drug with jak 3 is 2.42. The ligand interactions as derived from MOE software are as follows

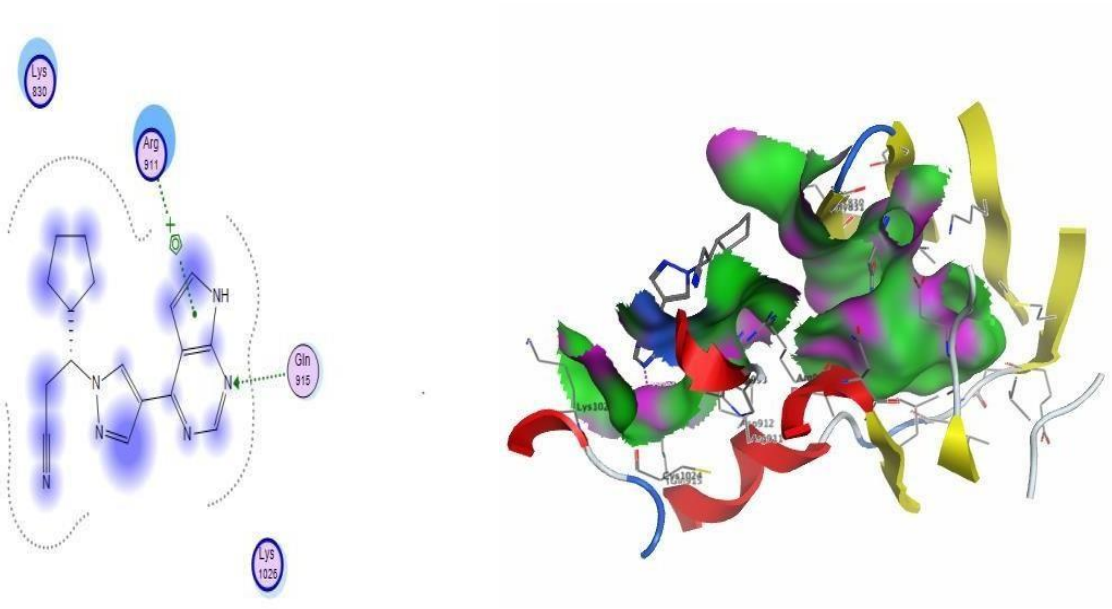


Figure 27 2-Dimensional interactions of docking of JAK3 with Ruxolitinib(left) and 3- Dimensional interactions of docking of JAK3 with Ruxolitinib from MOE

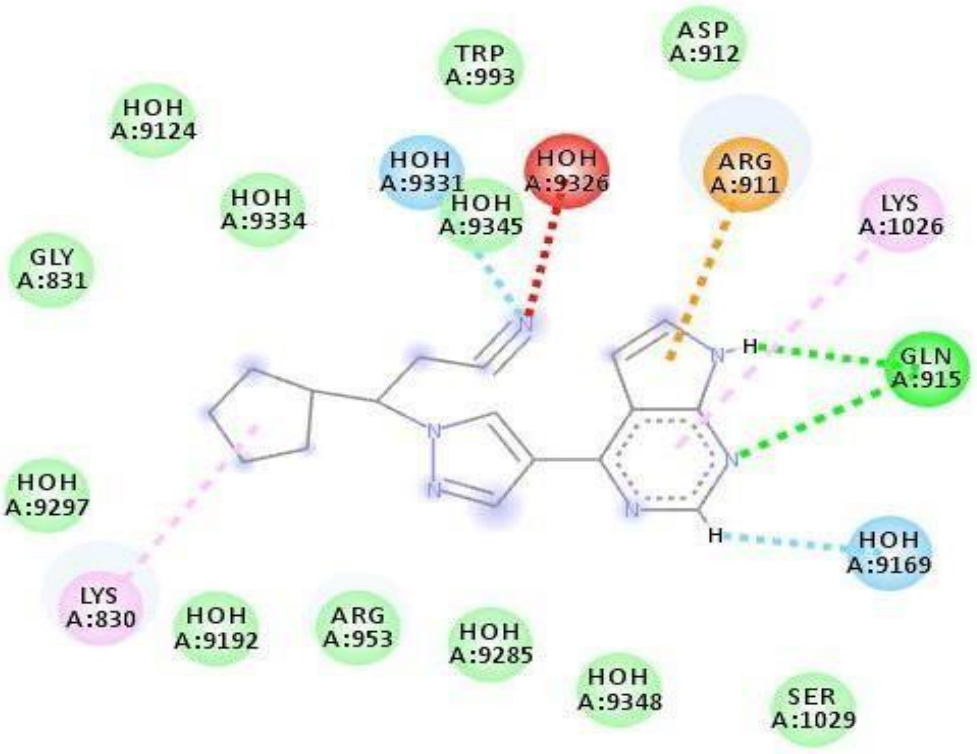


Figure 28 2-Dimensional Interactions of JAK3 with Ruxolitinib from Biovia

4.97 Docking PTGS1with1(3(Cyclohexylamino)propyl)guanidine

Docking Results of of PTGS1 with1(3(Cyclohexylamino)propyl) guanidine from cytoscape are shown in the following table

Table 13 Result of docking of PTGS1 with 1-(3(Cyclohexylamino)propyl) guanidine

S#	mol	mseq	S	rmsd-refine	E-Conf	E-place	e-score	E-refine	E-score2
1	**1-(3(Cyclohexylamino)propyl)guanidine**	**1**	**-9.33**	**2.70**	**6.23**	**-31.01**	**-11.39**	**-9.94**	**-8.77**

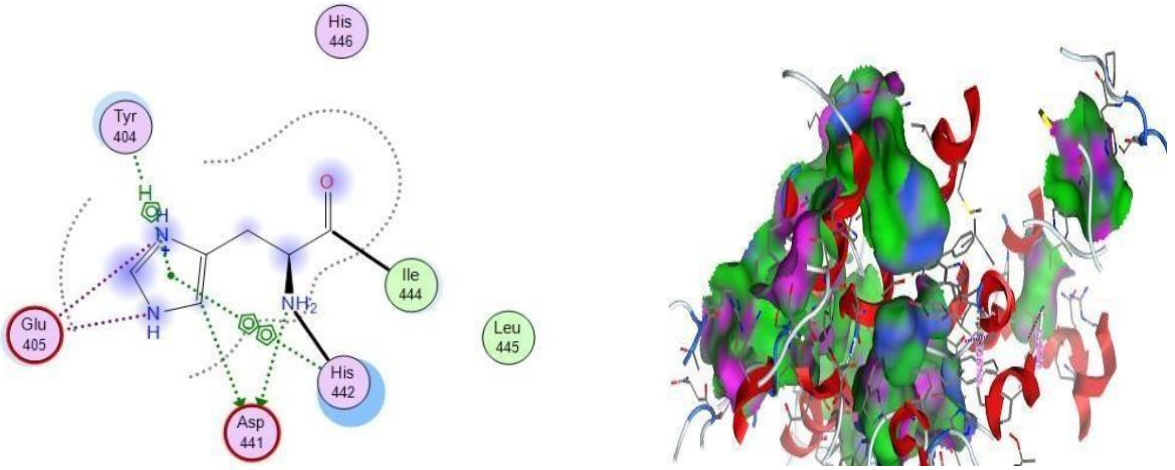


Figure 29 2-Dimensional interactions of docking of PTGS1 with with 1-(3(Cyclohexylamino)propyl)guanidine (left) and 3-Dimensional interactions of docking of PTGS1 with with 1-(3(Cyclohexylamino)propyl)guanidine from MOE

Chapter 5

Discussion

Rheumatoid arthritis (RA) is a complicated inflammatory illness with consequences that go far beyond joint inflammation. Recent research has shed insight on the illness's multiple consequences by elucidating several facets of the condition. RA being a T cell mediated autoimmune disease leading to vast annihilation of synovial lining, bone, and cartilage of joints in its patients globally (Victoria Ruffing, 2017). The systemic aspect of RA is becomingmore clear, with a growing body of literature pointing to links between chronic inflammationand cardiovascular problems. Recent research has found that RA patients have an increased risk of atherosclerosis, which leads to an elevated risk of cardiovascular events such as heart attacks and strokes. This complicated connection between immune dysregulation and cardiovascular health underscores the importance of a comprehensive therapeutic strategy The growing awareness of the impact of RA on organ systems other thanthe joints emphasizes the significance of targeted therapies. Precision medicine has the potential to detect high-risk individuals and personalize treatment solutions. Healthcare practitioners can lessen the wider consequences of RA and improve the overall quality of lifefor individuals affected by treating the extensive network of problems (Solomon, 2020).

The use of herbal medicine in the treatment of rheumatoid arthritis (RA) has received attention, indicating a move toward more holistic approaches to healthcare. Recent research has looked into the effectiveness of several herbal medicines in reducing RA symptoms and maybe slowing disease progression. Anti-inflammatory effects of herbal substances such as turmeric, Boswellia serrata, and ginger correlate with the immunological components of RA. Acupuncture and Ayurveda, for example, have earned attention for their ability to improve

pain alleviation and general well-being in RA patients. While promising, the use of herbal medicine requires extensive scientific testing to ensure safety, effectiveness, and consistent doses. Collaborations between traditional medicine practitioners and contemporary healthcare systems have the potential to change the future of RA therapy by incorporating evidence- based herbal remedies into clinical trials (Choudhury, 2023).

Current analysis depicts the prediction of targeted therapy of *Thymus serpyllum* and *Cassia angustifolia* against Rheumatoid Arthritis. In comparison with the already existing therapy, which due to its various side effects remains a worse problem itself, the natural compounds prove to be effective and nearly non – toxic as compared to current drugs.

Thymol, a phytochemical from *Thymus serpyllum*, has shown a very efficient activity against the rheumatoid arthritis target, Janus Kinase JAK on which the existing drug Ruxolitinib, with various side effects also binds predicting a good future possibility of treatment against Rheumatoid arthritis.

Since the plant extracts have shown good anti-inflammatory activity so it needs further validation by testing on animal models. As the study is directed to develop drug against rheumatoid arthritis, it would be of prime significance to verify the consequence of these drugs in living models of RA induced mice to establish the value of the extracts. The qualitative analysis of these extracts and further molecular identification may prove promising in developing drugs against a number of medical conditions, as there would be heightened effectiveness, lessened consequences and more conclusions that are favorable.

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Abstract

An autoimmune inflammatory disease, Rheumatoid Arthritis (RA) is affecting mainly joints in a symmetrical fashion stemming in inflammation, swelling and pain. Global mortality rate is 6 out of 1 million people undergoing the disease, whereas, in Pakistan it is 3 in every 1 million people. (HealthGrove)Natural compounds from various plants have been used for centuries to cure various diseases due to their less side effects. Rheumatoid Arthritis being a very arduous disease in terms of its treatment, needs improvement in its drugs. Organically obtained compounds can be one of the possibilities in medical treatment. In this study natural compounds from Thymus serpyllum and Cassia angustifolia were screened for their targets for Rheumatoid arthritis. By adopting the heterogeneous network clustering approach for natural compounds, the potential targets and drug target interaction was made to show the relationship between compounds and Rheumatoid Arthritis targets.

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