Computational Analysis of Untapped Natural Compounds of *Dodonaea viscosa* as a Potential Drug Target against Rheumatoid Arthritis

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

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I certify that this research work titled **"Computational Analysis of Untapped Natural Compounds of *Dodonaea viscosa* as a Potential Drug Target against Rheumatoid Arthritis"** is my work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred to.

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DEDICATION

*Dedicated to my beloved father, **Abdul Rehman Raja**, and my sweet mother, **Rehana Rehman**, for their unconditional support, love, and encouragement which helped me accomplish this milestone.*

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I am grateful to **Allah Subhana-Watala**, my Creator, for guiding me through every phase of my effort. Indeed, I would have been powerless without Your guidance and support.

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Sanaya Rehman

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LIST OF ACRONYMS

ACPA	Anti-citrullinated protein antibodies
ADME	Absorption, Distribution, Metabolism, and Excretion
Anti-CarP	Anti-carbamylated protein
BBB	Blood brain barrier
CADD	Computer-aided drug design
CTD	Comparative Toxicogenomic Database
DMARDs	Disease-modifying anti-rheumatoid drugs
DPPH	2, 2-diphenyl-1-picryl-hydrazyl-hydrate
FRAP	Ferric Reducing Antioxidant Power Assay
GC-MS	Gas Chromatography- Mass Spectrometry
HLA	Human leukocyte antigen
IL-6	Interleukin-6
MHC	Major Histocompatibility Complex
NSAIDs	Non-steroidal anti-inflammatory drugs
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RMSD	Root-mean-square deviation
ROS	Reactive oxygen species
STAT3	Signal Transducer and Activator of Transcription 3
TCM	Traditional Chinese Medicine

TFC	Total Flavonoid content
TNF	Tumor Necrosis Factor
TPC	Total Phenolic content
TPSA	Topological polar surface area

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ABSTRACT

Rheumatoid arthritis is an inflammatory condition that damages joints and cartilage over time. It results in swelling of the synovial lining, which makes movement difficult. Although the exact cause of RA is still unknown, it is well established that genetic and environmental variables have a role in its development. The condition is shown to affect females three times more frequently than males. The WHO estimates that it affects 0.3–1 percent of the world's population. In contrast, the incidence in Pakistan varies from 0.5 to 1.9 percent. There are numerous therapeutic options available for it, including NSAIDs, DMARDs, and biologics. These therapies aid in reducing disease-related symptoms, but their adverse effects have restricted their use. Thus, scientists are looking into herbal plants as a modern remedy for RA that has a high potential for treatment while minimizing side effects and is non-toxic, affordable, and cost-effective.

The therapeutic qualities of medicinal plants are derived from bioactive metabolites that help them fight pathogenic diseases. Similarly, *Dodonaea viscosa (L.) Jacq.*, an ethnobotanical plant, was used in this study to assess the effectiveness of its components in the treatment of rheumatoid arthritis. *D. viscosa* is widely distributed close to Margalla Hills in Pakistan. It is an evergreen, blooming hardy shrub with a variety of plant parts that are said to have anti-inflammatory, antibacterial, wound healing, and antioxidant effects.

Phenols, flavonoids, steroids, sterols, saponins, coumarins, tannins, and terpenoids were all detected in *D. viscosa* ethanol extract. The DPPH and FRAP experiments demonstrated good antioxidant activity. Similarly, *D. viscosa* extract exhibited significant anti-inflammatory potential in tests like the protein denaturation assay and the HRBC membrane stabilization experiment.

In silico studies revealed that nine of the 480 compounds found in *D. viscosa* ethanol extract had drug-like properties. TNF-α, STAT3, and IL-6 were found to be among the top three RA-related genes in a HUB gene analysis of the top 200 RA genes. The signaling pathways of these genes result in the activation of JAK/STAT, Nuclear factor κB (NF-κB), and Mitogen-activated protein kinases (MAPKs) pathways, respectively.

Significant findings were obtained from the molecular docking analysis of three protein targets with nine ligands. The compound 06, 4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl) benzoic acid, had the lowest binding score for TNF-α. Comparatively, compound 7 (3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-hydroxy-2H-isoindol-1-one) displayed the lowest binding energies for STAT3 and IL-6. The docked complex's highest stability is indicated by its lowest binding energy. The MD simulation findings for these three complexes showed that only compound 6-TNF complex remained stable, proving that compound 06, 4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl) benzoic acid, is a good small molecule inhibitor of TNF-α and may one day be employed as a potent drug.

The current study concludes that *D.viscosa* have excellent inhibitory potential against TNF-α, which plays a significant role in the disease. However, additional in-vitro and in-vivo testing is strongly advised to assess the efficacy of nine compounds in alleviating the effects of Rheumatoid arthritis progression.

1. INTRODUCTION

1.1. Rheumatoid arthritis (RA)

Rheumatoid arthritis is a chronic disease of synovial inflammation and disruption of joints, eventually leading to disability. Although the etiology of the disease is not yet known studies demonstrate a connection between RA with genetic susceptibility and environmental factors. The immune system plays a key role as it is an autoimmune disease, where the body's immune system begins to attack its cells considering them as a foreign agent. Therefore, resulting in synovial membrane inflammation, production of autoantibodies including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) along with the growth of abnormal tissue masses in joints, leading to articular cartilage destruction and bone erosion. Other factors that play a key role in the onset of the disease include age, a positive family history, silicate exposure, disease duration, smoking habits, immunological triggers, and psychological factors (Pankowski et al., 2022).

RA is one of the highly predominant inflammatory diseases ranging from 0.1% to 2% worldwide adult population. Studies demonstrate an increase in the disease prevalence with the increasing age, which affects both genders, whereas the female population is 2-3 times more susceptible to developing the disease (K. Almutairi et al., 2021; Markenson, 1991).

The goal of RA treatment is to minimize joint degeneration, and reduce bone erosion, and pain so that joints can continue to function. Since RA is a chronic condition, it requires lifelong care. Depending on the severity of the ailment, a variety of treatment methods are available. NSAIDs (non-steroidal anti-inflammatory drugs), DMARDs (disease-modifying anti-rheumatoid drugs), and corticosteroids are some of the medications used to relieve pain and inflammation in the joints. DMARDs are recommended as the first-line treatment of early RA (Wang et al., 2018).

Biologic drugs provide targeted therapy to reduce RA progression and are part of new treatment techniques. However, all of these therapeutic procedures have a variety of side effects and are expensive (*Anti-Arthritic And Anti Inflammatory Activity Of Beta Caryophyllene Against Freund's Complete Adjuvant Induced Arthritis In Wistar Rats | Insight Medical Publishing*, n.d.).

1.2. Epidemiology of RA

Epidemiology represents the distribution and determining factor of the disease in the general human population. Rheumatoid arthritis is one of the major forms of arthritis. It has various epidemiological features. The disease is less common among young people (age ≤ 18) whereas it has a higher ratio among the female population due to unexplained reasons (Gabriel, 2001; SILMAN, 1994).

The disease was first described by Cobb and his colleagues in 1953, and since then increased mortality rates have been observed in RA patients worldwide. It is now acknowledged that patients having rheumatoid arthritis are at about a 50% higher risk of premature death. Studies have shown that the life expectancy of such patients is decreased from 3 to 10 years as compared to the overall population.

Currently, there has been an ongoing debate about the incidence and prevalence of rheumatoid arthritis. Some studies have suggested that the evident rising rates of RA have started to decline in the mid-20th century. According to The National Arthritis Data Workgroup (NADW), the prevalence of RA in 2005 was 1.3 million U.S. adults whereas, in 1995, it was 2.1 million. A similar study by the Rochester Epidemiology Project (REP) reported that the prevalence in 2005 was 1.5 million U.S. adults. Between 1980 and 2019, the prevalence rate in the worldwide population was 0.46 percent, with industrialized countries having a higher prevalence than developing countries (K. B. Almutairi et al., 2021). According to (*Arthritis Statistics 2022: What Percent of the Population Has Arthritis?*, n.d.), around 350 million people have arthritis worldwide.

Based on these studies it is evident that the prevalence rate of RA has decreased in recent years but still, very few studies have been reported to support this evidence. Considering the above-mentioned hypothesis, the change in the trend of disease could be related to the underlying incidence rate of RA or changes in life expectancy (Kawatkar et al., 2019).

Environmental and genetic factors both play a key role in the progression of the disease. The descriptive epidemiology of rheumatoid arthritis demonstrates the genetic effects. Prevalence of different countries from European and North American populations has been reported suggesting that Native American populations show the highest prevalence of RA. Whereas, studies from Southeast Asia including China and Japan have shown low incidence rates from 0.2 to 0.3% (Silman & Pearson, 2002).

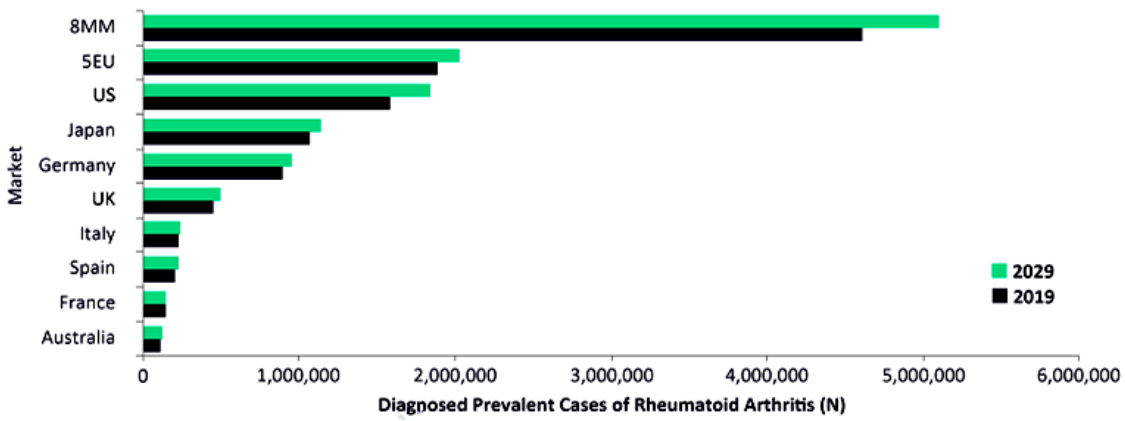


Figure 1.1. Epidemiology forecast from 2019 to 2029.

Source: (Rheumatoid Arthritis - Epidemiology Forecast to 2029, n.d.)

1.3. Pathogenesis of RA

Many studies have been reported on the immune-mediated pathogenesis of RA. It is an autoimmune disease that demonstrates the role of the innate and adaptive immune systems. Synovial injury, hyperplasia, and infection can cause tissues to produce cytokines like IL-6, IL-1, and TNF-α. When these cytokines are present in excess, they cause inflammation. This inflammation leads to synovitis which is the inflammation of

the entire joint capsule including synovial fluid, synovial membrane, surrounding tendons, and cartilage (*Rheumatoid Factor - Mayo Clinic*, n.d.).

Another destructing factor in RA is the production of reactive oxygen species (ROS). These are highly reactive, unpaired free radicals that function as irritating factors in multiple autoimmune diseases. Previous studies have shown a correlation between oxidative stress and autoimmune diseases. For example, nitric oxide (NO) is clinically proven to contribute to T cell dysfunction. Higher levels of ROS indicate the severity and progression of the disease. These are generated as a by-product of oxidative phosphorylation during electron transfer and also as a cellular response to cytokines, bacterial invasion, etc. (Sies & Jones, 2020).

They have several types including peroxide, superoxide, hydroxyl radicals, and nitric oxides (NO). Inflammation can trigger the production of ROS and elevated levels of several types of ROS lead to cellular and molecular damage also known as “oxidative stress.” These unpaired free radicals can affect cellular and extracellular components such as DNA, proteins, and lipids causing cartilage destruction and bone erosion (Veselinovic et al., 2014).

The clinical stages involved in the pathogenesis of RA include deposition of immune complexes in the synovium, intrusion of neutrophils leading to angiogenesis, and T cell activation. Eventually, it activates macrophages, allowing them to enter the cytokine-rich environment.

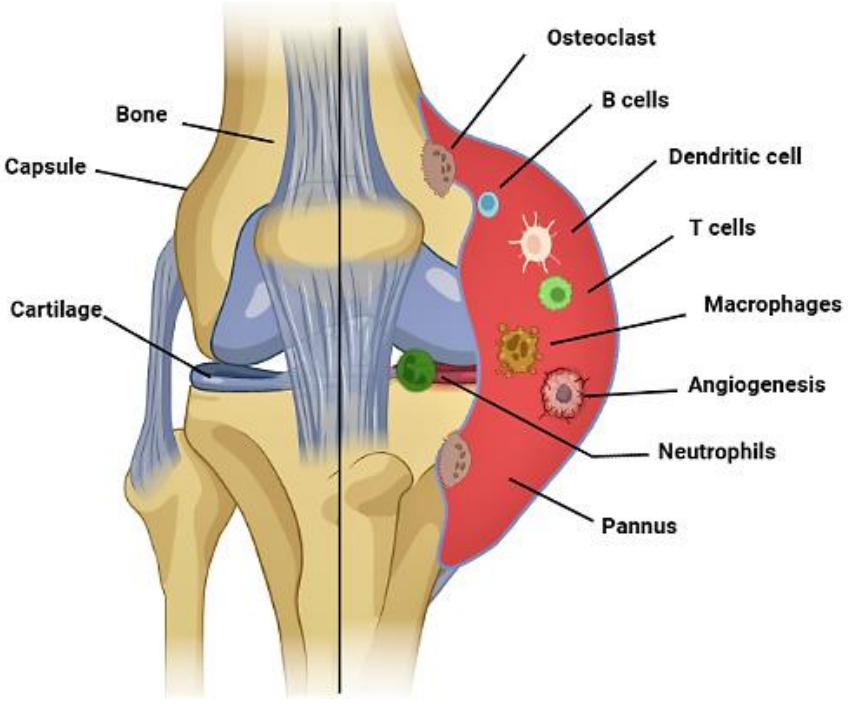


Figure 1.2. Pathogenesis of RA.

Adapted from (Aminpour et al., 2019); Created with BioRender.com.

1.3.1. Role of the innate immune system

When tissues are inflamed, the immune system comes into play. Immune cells play a crucial role in initiating and maintaining joint inflammation. These cells include dendritic cells (DC), macrophages, B cells, fibroblasts, T cells, neutrophils, and osteoclasts. When RA-specific autoantigens are not completely removed by the immune cells, more immune cells keep on activating and releasing higher levels of cytokines in the tissue, which results in a chronic inflammatory state in the joints. This state causes the synovial membrane to be inflamed causing pain and swelling. Furthermore, the synovial membrane extends and forms an abnormal mass which is termed a "pannus." This protruding mass grows into the bone-joint junction, following the destruction of cartilage and bone (Lin et al., 2020).

1.3.2. Role of the adaptive immune system

The adaptive immune system plays a significant role in RA pathogenesis since it is an autoimmune disease. Clinical studies demonstrate that the penetration of B cells in the synovium is related to the severity and prognosis of the disease. In the adaptive immune system, B cells are vital components. They are types of white blood cells responsible for providing humoral immunity, also known as "memory antibodies." Normally, these cells produce antibodies that help to fight against invading viruses and bacteria. Whereas in RA, some of these B cells present in the synovium divide into plasma cells which produce various autoantibodies while others differentiate into effector B cells, which produce pro-inflammatory cytokines and play a role in expressing RANKL. These synovial B cells play a significant role as antigen-presenting cells as well. Moreover, synovial T cells participate in initiating antibody production and local inflammation. CD4 and CD8 cells are the subsets of T cells involved in the production of pro-inflammatory cytokines like IFN-γ, TNF-α, IL-21, and CXCL13 that result in the pathogenesis of RA (Yamada, 2021).

1.3.3. Role of epigenetics in RA progression

Epigenetics plays a significant role in the pathogenesis of rheumatoid arthritis. It is the alteration of the gene activity without altering the DNA sequence. It is important for determining the expression or silencing of a gene which helps in the formation of various tissue-specific cellular phenotypes.

In RA patients, these epigenetic changes include DNA methylation, histone modifications, and expression of micro-RNA in immune cells as well as stromal cells. These pathways are thought to have an inflammatory role in the pathogenesis of RA. Some of the crucial changes are observed in the peripheral blood mononuclear cells of RA patients, where changes in DNA methylation are noticed. For example, abnormal methylation of cytosine is noted in the IL-6 promoter of peripheral blood mononuclear cells of RA patients. Later, this was associated with reduced transcription of IL-6.

Whereas, in IL-10, loss of methylation at cytosine resulted in higher expression of IL-10 in the same cells of RA patients (Ospelt et al., 2017).

1.4. Risk Factors causing RA

RA is a systemic multifactorial autoimmune disease. Presently, there is not much understanding about the mode of action of the risk factors contributing to RA. Therefore, many attempts are made to fully comprehend them in their development and progression of RA. Studies have mentioned both genetic and environmental factors along with their roles in the disease progression (Aho & Heliövaara, 2009).

Some of the most common factors include female sex, which could be due to the changes in their hormonal environment like pregnancy, menstrual cycles, and breastfeeding. Smoking has been mentioned repeatedly, this also includes passive inhaling of smoke. Diet plays a significant role, since having diets with low antioxidants and high caffeine increase the risk of the disease. Other environmental factors include exposure to infections and pathogenic organisms like Epstein-Barr virus (EBV) (Oliver & Silman, 2009).

Recent advancements in understanding the impact of risk factors in RA progression have been made possible due to the development of the seropositive RA model. Seropositive RA is a subtype of RA that has elevated serum levels containing autoantibodies; long before any preclinical symptoms of the disease appear, suggesting that the presence of circulating autoantibodies may have been many years before the onset of the disease symptoms. This duration is known as “Preclinical RA.” During this stage, genetic and environmental factors play their role in the propagation of autoimmunity and disease development.

1.4.1. Genetic risk factors

According to studies, genetics play a leading role in the development of RA. An increased prevalence of the disease within families leads to the familial risk of around 40 to 50% of seropositive RA, with the highest risk among first-degree relatives. The genetic

factors of rheumatoid arthritis have an increased prevalence within certain racial groups for example 5 to 7% of RA prevalence is exhibited by North American natives (Aho & Heliövaara, 2009).

Studies from familial and twin studies have supported the correlation of strong inherited components of RA, suggesting that around 60% of the susceptibility factor is due to genetic factors. The study demonstrated a high correlation among monozygotic twins. (Wordsworth & Bell, 1991). Moreover, it was also proved that RA is more prevalent in first-degree relatives (Junco et al., 1984).

Multiple gene-specific loci have been reported to play crucial roles in RA development. Major histocompatibility complex (MHC) is a group of genes or alleles that encode amino acids present on the cell surface, which play a vital role in the recognition of external pathogenic substances by the immune system. All higher vertebrates have MHC proteins whereas, in humans, this complex is also known as the human leukocyte antigen (HLA) system. These peptides help in predicting structural features of the HLA-peptide binding groove, collectively termed as "shared epitope" or SE (Gregersen et al., 1987; *Major Histocompatibility Complex | Genetics | Britannica*, n.d.).

Some of the major genetic factors associated with RA are as follows:

(i) Major histocompatibility complex (MHC) regions encoding HLA proteins have an elevated risk of RA-associated amino acids at positions 70 and 71.
(ii) DNA methylation alterations
(iii) Interleukin 6 receptor (IL6R)
(iv) Protein tyrosine phosphatase, non-receptor type 22 (PTPN22)
(v) Signal transducer and activator of transcription 4 (STAT4)
(vi) Tumor necrosis factor receptor-associated factor 1 (TRAF1)

All of these mentioned genetic factors play a vital role in increasing inflammation which is the hallmark of the disease. They also increase cell activation, citrullination, and higher antigen presentation. Therefore, triggering the development of rheumatoid arthritis (Ferreira et al., 2013; Stanford & Bottini, 2014; Van Der Woude et al., 2009).

1.4.2. Environmental risk factors

Apart from genetic factors, environmental factors also have a significant part in the development of rheumatoid arthritis. Among all these factors, cigarette smoking is one of the strongest and most consistent factors associated with RA patients, especially with seropositive RA phenotypes. It is reported that smoking is associated with 25% of overall RA and 35% of seropositive type RA (Costenbader et al., 2006; Källberg et al., 2011).

Some of the common environmental and other factors which are associated with rheumatoid arthritis include:

(i) Exposure to cigarette smoke
(ii) Female sex
(iii) Exposure to silica dust, air pollution, ultraviolet (UV) light exposure
(iv) Obesity, high birth weight
(v) Low levels of vitamin D in the body
(vi) Alterations in the intestinal microbiome have been identified recently and ongoing studies are continuously investigating them as well.
(vii) Breastfeeding, pregnancy, and low alcohol intake
(viii) Stress and psychological triggers

Among these, some factors have robust evidence while others have moderate to least evidence for association with higher RA risk. Exposure to tobacco smoke is one of the strongest factors. Whereas hormonal and dietary factors have shown the least risk concerns (Karlson & Deane, 2012; Lahiri et al., 2012).

RA is three times more common in females than in males, indicating that hormonal factors have a key role in its etiology. One study suggested that the risk of RA was increased by breastfeeding due to the higher production of prolactin which is a pro-inflammatory hormone. Another study indicated comparable results demonstrating that the initial stages of breastfeeding produce a prolactin surge which triggers RA in

susceptible individuals. However, prospective studies have supported the protective role of breastfeeding and antioxidants as well (Lahiri et al., 2012).

It is therefore concluded that environment and lifestyle can influence the risk of developing RA however the risks may vary from person to person. Some factors like smoking alcohol and coffee are associated with seropositive disease. Whereas others like obesity and breastfeeding may be associated with negative diseases. Further, environmental, and genetic factors both have some common interactions in the development of RA. Understanding these risk factors and their associations will help to manage and minimize the disease in the future (Aho & Heliövaara, 2009).

1.5. Diagnosis of RA

The diagnosis of rheumatoid arthritis (RA) is an important struggle for clinical rheumatologists, due to increasing evidence that preliminary treatment with therapeutic medicines like disease-modifying anti-rheumatic (DMARDs), improves disease outcomes. Early diagnosis helps to determine whether clinical data is important and contributes to the assessment of diagnostic potentials. Unfortunately, the diagnostic research on RA it's not sufficient enough due to which a huge gap between the results of diagnostic research and the testing procedure in a practical clinical setting exists (Visser, 2005).

Therefore, diagnostic investigations are essential to generate diagnostic criteria or prediction models that allow clinicians to distinguish RA from other bone ailments in the preliminary stages of the disease.

The most common gold standard for the diagnosis of RA was published in 2010 by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR). According to this, there are two mandatory criteria. First, the patient should have at least one joint actively involved in synovitis. Second, the criteria are only applicable to those in which synovitis is not explained by any other disease diagnosis, for example, lupus, gout, etc. Additionally, four criteria are applied to these

patients with their indicative scores for the presence of RA. The score can range from 0 to 10, where a score of ≥6 indicates the presence of definitive RA. These remaining criteria include joint involvement, serology test, acute phase reactants, and duration of the symptoms. If a person cannot meet the required criteria, they are not classified as having absolute RA (Aletaha et al., 2010).

Table 1.1. 1987 classification criteria for RA. (Aminpour et al., 2019)

Criterion	Explanation
Morning stiffness	It starts at least one hour before maximum improvement
Arthritis of small hand joints	At least one swollen joint of the hand
Arthritis in three or more joints	At least three joint areas/ joints are swollen
Symmetric arthritis	Involvement of bilateral joints or symmetrical swelling of joints
Rheumatoid nodules	Subcutaneous nodules present in bony or articular regions
Serum rheumatoid factor (RF)	Abnormal amount of serum rheumatoid factor
Radiographic changes of RA	Radiographic changes of RA on hand and wrist radiographs

Clinical features for the diagnosis

Rheumatoid arthritis does not exhibit a well-defined natural history, therefore, making it difficult to predict its prognosis. The clinical presentation of the disease varies from person to person but symmetric swelling, pain in joints anorexia, and lethargy are the most common findings. Early symptoms of the disease are more generalized including fever, fatigue, and weight loss. Early RA can be described as symmetric polyarthritis typically involving small joints of the wrist, hands, and feet.

RA has variable clinical manifestations, depending upon the joint involved and the stage of the disease. Synovitis is the inflammation of the synovial membrane, an early clinical symptom of RA patients. People with RA have often reported morning stiffness in and around the joints that last for at least one hour, pain, and swollen joints.

In the initial stages, hand, wrist, and foot joints are the hot spots for systematic expression of RA. In hand joints, it causes a distinctive painful swelling and severe immobility, with no noticeable radiologic sign of bone destruction. Whereas, in the foot, metatarsophalangeal joints develop synovitis at an early stage of the disease development. This area has shown clear radiographic changes including, bone decalcification and erosion in the early onset of the disease. Other complex deformities can occur at later stages of the disease (Grassi et al., 1998a).

In later stages of disease progression, knee joints of around 70% to 80% of RA patients are targeted by the disease, resulting in functional impairment and decreased quality of life. Hip joints are rarely affected by the disease but if they are involved, they can result in severe disabilities in 50% of the patients. Shoulder joints can be affected in either early or later stages of the disease. As the disease has slow continuous progression with high remission rates, the symptoms are often misunderstood and ignored. Therefore, a detailed history and careful laboratory analysis with radiological findings are necessary to efficiently diagnose the disease at an early stage (*Diagnosis and Management of Rheumatoid Arthritis - American Family Physician*, n.d.).

1.5.1. Serologic testing

Standard laboratory tests may contribute to the confirmation of the diagnosis and thus the determination of the level of inflammation, autoantibodies, and the potential damage or erosions.

Autoantibodies are often used to help diagnose autoimmune disorders in the past. Serologic marker testing contributes to enhancing diagnostic rates for the assessment of reactive polyarthritis. Production of autoantibodies is the hallmark of the disease, including multiple factors. These function as a diagnostic marker of the disease, indicating disease activity, severity, and joint damage.

Among these vital players, serum rheumatoid factor (RF) is associated with RA. Rheumatoid factors (RF) are proteins that can attack the body's healthy tissues. Elevated levels of RF have been detected in 60% of patients with rheumatoid arthritis. This association is not clear enough, but it has been reported in multiple autoimmune diseases. RF should not be considered as a screening tool since it is present in 5% of other conditions as well like, chronic liver disease, hepatitis, leprosy, and mycobacterial disease. Whereas in the case of unexplained polyarthritis with swollen joints, it should be ordered wisely (van Delft & Huizinga, 2020).

The second factor, anti-citrullinated protein antibodies (ACPA) is present in 50% of RA patients and is said to have a diagnostic and predictive role in RA. ACPA-positive patients have shown more severe disease symptoms as compared to ACPA-negative RA patients. This gives rise to a hypothesis that ACPA might be pathogenic itself (Szodoray et al., 2010).

Thirdly, the presence of anti-carbamylated protein (anti-CarP) antibodies also plays a key role in the diagnosis of RA many years before the first clinical symptom appears (Cush, 2021; Shi et al., 2014).

1.5.2. Biomedical imaging

Early diagnosis of RA is crucial for understanding and managing the disease effectively. Biomedical imaging techniques provide valuable information in detecting inflammatory changes in the joints. There are many biomedical imaging techniques including X-rays, CT scanning, MRI scanning, and many more (McQueen, 2013).

1.5.3. Radiography (X-Ray)

Radiographs have significant advantages over traditional RA assessment methods, such as tender or swollen joints. X-ray reflects the background of joint pathology. Since the damage observed on X-rays is irreparable, this marks the long-term inflammatory process. Furthermore, they give a permanent record that can be used to track the condition over time. A major drawback of radiographs is that quality and stability issues can arise during the technical production of radiographs which may result in problems with diagnosis (Van der Heijde, 1996).

1.5.4. Magnetic resonance imaging (MRI)

In the early diagnosis and treatment of RA, magnetic resonance imaging (MRI) is the preferred technique. When it comes to detecting inflammatory responses in the joints, MRI has a high sensitivity, strong reliability, and minimal fluctuation. According to several studies, MRI can detect and quantify synovial abnormalities. The use of a contrast agent improves sensitivity in detecting erosions that distinguish synovial growth from the fluid collection. MRI aids in the early diagnosis of RA which facilitates immediate treatment with various medicines and therapies (Tehranzadeh et al., 2003).

1.5.5. Computed Tomography (CT)

CT is better than MR imaging for studying bones because it can detect more minor changes in the bone cortex. However, it cannot detect inflammatory processes like synovitis. CT scans are more accurate than other methods for detecting bone erosion.

They allow careful examination of bone damage. The most recent CT scanners can detect even minor bone changes. As a result, they may be considered the gold standard for bone erosions, even though radiation exposure causes a major hurdle to everyday use (Barile et al., 2017).

All these techniques help in the early diagnosis of RA, which is significantly important in managing and treating the disease add initial levels.

1.6. Treatment for RA

The primary goal of treatment is to slow the progression of the disease, minimize inflammation and joint damage, as well as enhance the patient's quality of life. There is currently no effective treatment for RA available. However, over the last two decades, treatment alternatives have vastly improved, with new medications offering enormous support and relief for individuals in the preliminary stages of RA. Any therapy or medication that promises to treat RA should be considered with caution (Nell et al., 2004).

Treatment in the initial stages of the disease is important since delay in therapy beyond 90 days can result in irreparable damage to joints. Bone erosion can be seen in 25% of patients within the first three months of disease progression. Whereas 70% of bone erosion is evident within three years duration if left untreated. this can lead to permanent loss of mobility decreasing the quality of life along with pain swelling and other symptoms (Finckh et al., 2006).

RA is an autoimmune illness that results in abnormal immunological and inflammatory processes associated with destructive mechanisms. The primary goal in the treatment of disease is to stop the inflammation to manage disease progression. Therefore, the use of disease-modifying anti-rheumatic drugs (DMARDs) such as leflunomide, methotrexate (MTX), and sulfasalazine (SSP) have shown impressive results in reducing disease progression which could lead to it irreparable joint destruction.

1.6.1. Early Treatment Concepts

In past, many methods have been used to treat and manage rheumatoid arthritis including leeching, cupping method, also known as Hijama (Mayberry, 2017), acupressure, and acupuncture (Joo & Park, 2017), use of heat, use of heavy metals like copper salts arsenic and gold. These were some of the methods commonly prescribed for the management of disease (J. Zhang et al., 2017).

Non-pharmacological treatment of RA

Diet and nutrition play a considerable role in the management of diseases by relieving symptoms and decreasing the progression of the disease. Due to the danger of developing osteoporosis in people with RA, nutritional management is critical. However, people consume enough calcium daily from food to maintain physical fitness and strong bones. Moreover, green vegetables, cheese, yogurt, salmon, almonds, and calcium-fortified drinks are all good sources of calcium and are included in the diet regularly (Van Der Linden et al., 2010).

Similarly, a balanced diet rich in fruits, cooked vegetables, fish, and olive oil has been strongly recommended as a preventive measure against RA due to elevated concentrations of omega-3 fatty acids in such diets (Ariza-Ariza et al., 1998; Rennie et al., 2003).

Many studies have reported improved RA and inflammatory symptoms by the consistent use of fish oils. These help in the reduction of swelling and tenderness in the joints. Studies since 1985, demonstrate significant clinical improvements by dietary supplementation of fish oil in RA patients (Darlington & Stone, 2001; Simopoulos, 1999).

1.6.2. Current Treatment Approaches

Major advancements in the past decade have paved the way for understanding rheumatoid arthritis, providing better management and treatment options (Davis & Matteson, 2012).

Since the 1980s, RA treatment options are dramatically changing. Progressive built up from a foundation of physical and non-pharmacological therapies, followed using aspirin and other NSAIDs, and finally the initiation of single DMARDs. These would be maintained for a prolonged period without any side effects. Later, multiple combinatorial DMARD therapies were considered effective in the treatment of RA. The emergence of methotrexate as the first DMARD helped in the advancement of RA treatment options (van Vollenhoven, 2009).

1.6.2.1. NSAIDs

As rheumatoid arthritis is an inflammatory disease, therefore, initially the key focus is to suppress the inflammation mechanism. For that purpose, firstly, nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin and glucocorticoids were used to minimize pain and swelling of joints in RA patients. This is among the most commonly utilized clinical therapy options for the treatment of RA. These are excellent anti-inflammatory and analgesic medicines due to their capacity of blocking prostaglandin synthesis at the level of the cyclooxygenase enzyme. Since various side effects of NSAIDs are linked to prostaglandin suppression, therefore, it is significantly important to understand the biology of prostaglandins and their link to pharmacologic features of various NSAIDs (Crofford, 2013).

1.6.2.2. DMARDs

Disease-modifying anti-rheumatic drugs (DMARDs) are the next class of drugs used against RA. They include methotrexate, sulfasalazine, and leflunomide. These are slow-acting compounds with a higher effectivity rate. But their onset time can vary from weeks

to months before showing any clinical and radiological improvements. On the other hand, NSAIDs and glucocorticoids act rapidly, hence acting as a bridge when utilizing DMARDs in treatment (Gaffo et al., 2006).

Methotrexate is the first DMARD used for almost 20 years. It has been reported for its effectiveness, reliability, low cost, high tolerability, and low mortality rate. It suppresses the release and action of Il-1, Il-8, and TNF-α, which results in lower blood levels of Il-6. All these changes restrict the proliferation of macrophages, monocytes, and synoviocytes. Thus, methotrexate inhibits leukocyte movement to tissues by reducing the expression of adhesive proteins in epithelial cells and the synthesis of cytokines and chemokines. Despite being the number one drug at that time, it still had some major drawbacks. Since methotrexate is a folic acid anti-metabolite, exhibiting cytostatic and immunosuppressive potentials, therefore, its cytotoxicity had to be taken into consideration before use. Another drawback was that it had embryotoxic properties due to which it was not recommended during pregnancy. Moreover, it affects female fertility and causes temporary oligospermia in males. Therefore, new alternatives with fewer side effects were required (Wiewiórowski & Graczyk, 1999).

1.6.2.3. Biologic drugs

Biologic drug agents are a novel class of rheumatoid arthritis medicines (RA). They have been genetically modified to mimic the functions of natural proteins in innate immunity to create biologic disease-modifying anti-rheumatic medications (bDMARDs). Biologics do not cure RA, although they can help it evolve more slowly. They also have fewer adverse effects than other types of medications (*Biological Treatments for Rheumatoid Arthritis*, n.d.).

1.6.3. Importance of medicinal plants/ herbal medicine

Plants and herbs have been used in ancient times to treat diseases. They were mostly used in crude form. The concept of herbal medicine has allowed us to understand the relationship between man and his environment. Humans had been more focused on the

development of chemically synthesized drugs for their beneficial biochemical constituents involved in treating various diseases. But due to their mild to severe side effects, they are thought to be less ideal nowadays.

Researchers are exploring for alternative therapy to manage pathogenic disorders to reduce these negative effects. These include herbal medications, which are synthesized from natural herbal plants having therapeutic characteristics. Many medicinal plants have been found to possess natural compounds that can be employed to cure a variety of diseases (Rehman, 2021).

Medicinal plants are widely employed as raw resources for extracting active components that are used in the production of various medications. Plant-based compounds are found in laxatives, antibiotics, and anti-malarial treatments. For example, Taxol and morphine are active compounds extracted from foxglove and opium poppy, respectively.

There are more than about half a million plants president the world, and most of them have not been explored yet. Hence, medicinal plants can be investigated for their medicinal potential and can be used to send a size more effective drugs in the treatment of numerous diseases with negligible side effects (Rasool Hassan, 2012).

1.6.4. In-silico drug designing

Computer-aided drug design (CADD) approaches have become highly significant in drug development, and they are essential in identifying promising therapeutic candidates at a low cost. These computational methods are useful for minimizing the use of animal models in pharmacological research, assisting in the targeted delivery of novel and safe drug candidates. These approaches also help to stabilize marketed drugs (Brogi et al., 2020).

A trend toward using in-silico chemistry and molecular modeling for CADD has rapidly received attention. Several software's are utilized in in-silico drug designing, grid computing, JAVA, PyRx, and Python. Multiple techniques are applied for in-silico drug designing, visualization, energy minimization, molecular docking, QSAR, etc.

Many important processes are conducted during the identification of emerging drug candidates to exclude compounds that have adverse effects. In the biotechnology and pharmaceuticals field, in-silico drug design software plays a critical role in the development of novel proteins and medications. Moreover, drug design tools are used to explore genetic molecular modeling, genomic regulation and analysis, and the three-dimensional structure of proteins.

Computer-aided drug design (CADD) approaches help in the target identification and prediction of novel remedial drugs (Brogi et al., 2020).

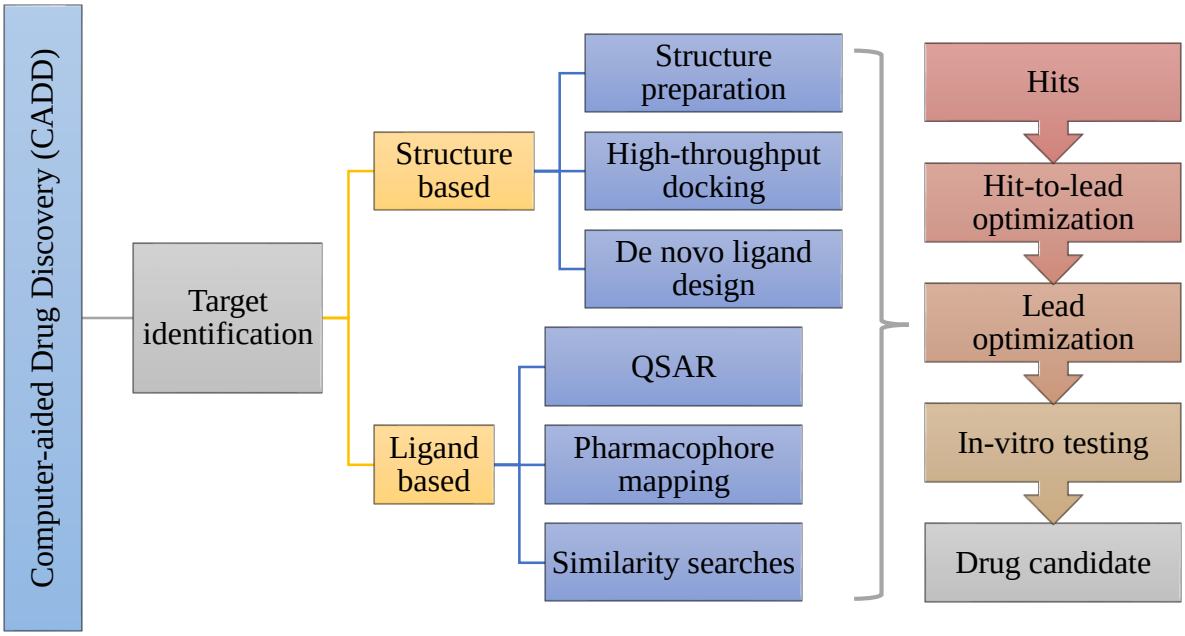


Figure 1.3. Schematic representation of a computer-aided drug design (CADD) pipeline. Adapted and modified from (Aminpour et al., 2019).

AIMS AND OBJECTIVES

1. To evaluate the therapeutic potential of *Dodonaea viscosa* against Rheumatoid arthritis.
2. Identifying and evaluating the pharmacological compounds present in the selected botanical source against Rheumatoid arthritis.
3. Identification and analysis of biologically active targets of RA and its pathways involved in disease pathogenesis.

2. LITERATURE REVIEW

2.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a progressive, chronic autoimmune disease that affects the lining of synovial joints, and they are associated with joint pains, inflammation, disability, and premature death. (Guo et al., 2018)

The clinical features vary from person to person depending upon the severity and spread of the disease. The initial sign of disease includes morning stiffness which indicates disease activity. Hand joints are the most common signs of RA. These include metacarpophalangeal, interphalangeal, and wrist joints with painful swelling that causes difficulty in hand movement. Fever, malaise, weight loss, and fatigue are among frequent clinical signs of RA. In later stages, progressive loss of articular cartilage in synovial joints is an obvious sign of RA. There are no direct tests for the diagnosis of RA therefore, different strategies are applied. Medical imaging techniques, such as X-ray, magnetic resonance, and computed tomography provide significant details of the extent of bone damage. Laboratory tests, such as erythrocyte sedimentation rate (ESR), C - reactive protein, hemoglobin, and platelet count are used to monitor rheumatoid arthritis and detect its negative effects. (Grassi et al., 1998b)

Rheumatoid arthritis is highly prevalent in many parts of the world. The prevalence of RA is 1% worldwide, whereas the western population has the highest prevalence of 1% and 2%. (Sultana et al., 2020)

According to a study in 2011, prevalence rates in Pakistan range from 0.5% to 0.7%. Karachi is among one of the big cities in Pakistan and the rate was 0.14%, with 0.55% in Northern Karachi. There have only been a few studies on RA and OA in Pakistani populations. (Naqvi et al., 2017)

Table 2.2. Studies report the prevalence (%) of RA in Pakistan. (Naqvi et al., 2017)

Author/ year	Venue	Year of study	Prevalence (%)
Shamim et al., (2015)[18]	Jinnah Postgraduate Medical Centre	August 2013 - January 2014	26.9%
Rais et al., (2014)[17]	Liaqat National Hospital	September 2006 - September 2012	21.7%
Alam et al., (2011)[16]	Liaqat National Hospital	January 2005 - June 2007	12.9%

According to numerous studies, environmental, genetic, and epigenetic factors play a contributing role in disease pathogenesis. These risk factors include susceptible genes (human leukocyte antigen (HLA) class II and many susceptible loci such as PTPN22, TRAF1, and CTLA4), non-genetic variables (smoking, dental infection, and microflora), immunologic (macrophages, dendritic cells, mast cells, neutrophils, T cells, and B cells) and nonimmune (fibroblasts and chondrocytes) cells, and inflammatory cofactors (autoantibodies, cytokines, and proteolytic enzymes) are collectively involved in the systemic inflammation targeting the bone and cartilage causing loss of joints. (Fang et al., 2020)

Many treatment options are available including conventional DMARDs, NSAIDs, steroids, biologic agents, and targeted synthetic DMARDs. But they all have mild to severe side effects. Therefore, alternative medicine is required to minimize them. (*Rheumatoid Arthritis - Diagnosis and Treatment - Mayo Clinic*, n.d.)

2.2. Need for Herbal Medicine for RA treatment

RA is a disease with no cure, but the disease can be managed and controlled with the right treatment options. There are many therapies which include DMARDs, NSAIDs, biologics, etc. These are sometimes prescribed individually or in combinations, depending on the severity and extent of the disease. With all the benefits, come the side effects that are impossible to neglect. Therefore, herbal medicine is considered a desirable alternative for combating inflammation-related diseases. Herbal medicine has been used as a natural source of treatment since ancient times. Herbal medications are quite important in sustaining one's health. The need for Herbal medicines is expanding in

both developed and developing countries due to growing awareness of natural plants being a negligible side effect, easily available in surrounding places at low cost. Various active elements can be found in different areas of the plant, and the level of activity and concentration of these active constituents can vary. These possess many properties such as antioxidant, anti-inflammatory, antimicrobial, anti-rheumatic, analgesic, etc. The majority of active components are found in the plant's leaves, flowers, fruit, bark, root, and seeds. Over the last few decades, several plants have gained popularity, but herbal remedies have remained unclear by the public and medical professionals. Therefore, strong phytochemicals of plants can be utilized in the treatment of various autoimmune diseases including rheumatoid arthritis. (Lawal & Yunusa, 2013)

2.3. *Dodonaea viscosa*

2.3.1. Physical and botanical description of *Dodonaea viscosa*

In the 16th century, a Flemish botanist named Robert Dodoens worked on *Dodonaea viscosa*, and it was named after him for his great contributions to the progress of science. Dodonaea is a genus that has over 70 species and numerous subspecies are extensively distributed around the world. *Dodonaea viscosa (L.) Jacq*. is a natural species used in traditional medicine that belongs to the Sapindaceae family. It is an evergreen blooming hardy shrub with a variety of plant parts. Early European Australians used to make a bear with *D.viscosa* and that is why it is commonly known as called **Hop bush**. (]) D Khan & Ismail, 2019). In the Urdu language, it is known as **Sanatha**. It has anti-inflammatory and analgesic capabilities due to its active chemical ingredients. Antioxidant and wound-healing properties have also been discovered in studies, which may aid in the formulation of pharmaceutical medications. (Beshah et al., 2020)

Figure 2.1. Images of *Dodonaea viscosa Linn*.

Table 2.2. Taxonomy of *Dodonaea viscosa*.

Kingdom	Plantae
Division	Tracheophyte
Class	Magnoliopsida
Order	Sapindales
Family	Sapindaceae
Genus	Dodonaea Mill.
Species	*Dodonaea viscosa (L.) Jacq.*

2.3.2. Traditional uses

Dodonaea viscosa has remarkable medicinal and therapeutic properties including both traditional and modern applications. It has been used as a folk remedy against many health disorders such as rheumatoid arthritis and joint pain. Due to the presence of various therapeutic properties, various parts of the plant are used as antiviral, analgesic, anti-inflammatory, anti-microbial, anti-ulcerogenic, and as laxatives. (“Antibacterial and

Antifungal Activity of *Dodonaea viscosa* (L.) Jacq., a Wild Plant of Azad Jammu and Kashmir," 2013)

Dodonaea genus has been traditionally used to treat many health disorders such as malaria, scurvy, inflammation, renal pains, sore throat, gastrointestinal parasites, herpes, rheumatism, asthma, backache, toothache, skin infection, tumor, wound healing, gout, hemorrhoids, pharyngitis, and many other ailments. Plant leaves, stems, and fruits are occasionally utilized in traditional Chinese medicine formulations. (Beshah et al., 2020). According to a study, leaves of *D.viscosa* act as smooth muscle relaxants. (Rojas et al., 1996)

2.3.3. Pharmacological properties

D. viscosa is a natural wild plant that has been used to treat a variety of ailments for centuries. Various parts of this plant are used to cure various conditions. Due to its long history of use, many scientific studies have been conducted to investigate the biological activity of plant extracts. (Hossain, 2019a)

Some studies have discovered physiologically active chemicals and used diverse models to assess their bioactivity. Previous studies have demonstrated that all parts of *D. viscosa* and its extracts have pharmacological potential. Many in vitro studies have been conducted for the presence of flavonoid content in *D. viscosa* leaves. (Al-Aamri & Hossain, 2016; AL-Oraimi & Hossain, 2016a)

Moreover, potential pharmacological activity can be due to one or more chemical constituents present in plant extracts. Therefore, multiple studies have been conducted on isolated phytochemicals indicating their role in the potential activity. (Abdel-Mogib et al., 2001; Dimbi et al., 1985; Sachdev & Kulshreshtha, 1984; Wabo et al., 2012)

2.3.4. Anti-inflammatory activity

Inflammation has been linked to cancer, rheumatoid arthritis, and hypertension, among other chronic degenerative disorders, contributing to high rates of death and morbidity all over the world. Therefore, many studies have discussed the anti-inflammatory potential of *D. viscosa.*

An important study discusses Hautriwaic Acid, a diterpene molecule derived from *Dodonaea viscosa*, which exhibited remarkable anti-inflammatory effects in TPA-induced mice. Mice were given different doses of this chemical, and the anti-inflammatory activity increased as the dosage was increased. When compared to the standard chemical (indomethacin), Hautriwaic Acid had a considerable inhibitory effect on swelling in chronic inflammation. The study demonstrated that *Dodonaea viscosa* can be a potential anti-inflammatory source. (Dimbi et al., 1985)

In another study, bioactive constituents were studied, and molecular docking simulation was used to assess Nebrodenside A, a molecule derived from *D. viscosa*, as a possible anti-inflammatory drug. When compared with the standard drug (diclofenac), Nebrodenside A proved to be significant in reducing inflammation. (Dimbi et al., 1985)

According to a study conducted in 2013, *D. viscosa* contains many bioactive metabolites, among which Viscosine, a flavonoid possesses a significant anti-inflammatory potential. Viscosine exhibited considerable lipoxygenase inhibitory potential. Where Lipoxygenase enzyme engages in fatty acid metabolism, and the metabolites it produces have anti-inflammatory properties. The key factor involved in the strong lipoxygenase inhibitory activity of viscosine appears to be its hydrogen-bonding interactions with the catalytic triplet (His523, His518, and Ile875) inside the active region of lipoxygenase. (A. Z. Khan et al., 2013). Among various types of extracts, studies have suggested that methanol extract and chloroform fraction have significant anti-inflammatory activity. (Mahadevan et al., 1998)

2.3.5. Antioxidant activity

The production of excessive reactive oxygen species (ROS) causes many health-related disorders. Therefore, antioxidants play a beneficial role in disease management. *Dodonaea viscosa* has been reported to have a lot of antioxidants in it. Several investigations have shown a dose-dependent response, with the total antioxidant capacity increasing as the concentration of the plant extract increases. Studies have suggested that methanol extracts of *Dodonaea* have higher DPPH radical scavenging activity as

compared to aqueous extracts. (*View of Phytochemical, Antibacterial, and Antioxidant Activities of Dodonea Viscosa Jacq. Extracts Cultivated in Iraq*, n.d.)

In another investigation, the human breast cancer cell line MCF-7 was used to assess the anti-cancer properties of *Dodonaea viscosa*. The antioxidant activity increased with the increasing plant concentration. The results revealed that the two variables had a linear relationship. Moreover, the researchers concluded that chloroform extracts of *Dodonaea viscosa* have antioxidant and anti-cancerous properties and therefore, they can be used in disease treatment. (Jayaraman et al., n.d.)

Methanol and chloroform extracts of *D. viscosa* flowers were used in a recent study. Both extracts of *D. viscosa* flowers were evaluated for their ability to protect mice from CCL4-induced toxicity. The primary objective was to use to identify phytochemicals, antioxidants, and anti-tuberculosis activities using GC-MS, HPLC, and FT-IR techniques. The study demonstrates that the antioxidant compounds in the plant are responsible for the plant's therapeutic effect against oxidative damage in the liver, kidney, and spleen. (Jayaraman et al., n.d.)

2.3.6. Anti-microbial activity

Different methods have been used to determine the growth inhibition of *Dodonaea viscosa* extracts against various microbes. These studies have been conducted on many extracts including hexane, water, butanol, ethyl acetate, chloroform, and methanol. These *D. viscosa* extracts have demonstrated significant antimicrobial activity against a variety of human infective bacterial strains, both Gram-positive and Gram-negative. (Hossain, 2019b)

According to a study, at most concentrations, all crude extracts demonstrated antimicrobial activity against cultured bacteria used. Among the six tested crude extracts of *D. viscosa* leaves, water crude extract had the highest potential antimicrobial capacity against protozoa (AL-Oraimi & Hossain, 2016b).

Previous studies have shown that the key determinants of the inhibition of growth include the bacterial strains and the incubation period. Various studies have discussed the use of

bacterial strains and their results on different extracts of *D. viscosa* proving that the plant possesses good antimicrobial potential. (AL-Oraimi & Hossain, 2016b)

2.3.7. Cytotoxic activity

Dodonaea viscosa has been reported to have a cytotoxic effect on various diseases. Among these findings, one investigational study reported the cytotoxic effect of *D.viscosa* on the colon cancer cell line (HT-29). In comparison to other fractions, the ethanol extract of leaves had a significant inhibitory effect on human colon cancer cells (HT-29). Moreover, the study concluded that *D. viscosa* has no cytotoxic effect on mouse epidermal cells (3T3), whereas slight cytotoxic effects on HT-29 when compared to standard (5-FU). Therefore, this plant can be an effective source of herbal treatment for colorectal cancer (Herrera-Calderon et al., 2020a).

2.4. Network pharmacology and Drug Designing

Drug designing is the process of investigating and finding new medicines based on previous knowledge of the biological target. The key components for drug designing are biological disease targets and bioactive plant compounds. With the advancement in research, the concept of "one-target, one-drug" has been shifted to "network-target, multiple-component-medicine." Similarly, network pharmacology is a method for determining "compound-protein/ gene-disease" networks. This concept has been adapted from Traditional Chinese Medicine (TCM). These networks are beneficial for understanding the behavior of small molecules in a high-throughput manner. Therefore, this approach is powerful for analyzing drug combinations (R. Zhang et al., 2019).

Moreover, the approach of network pharmacology is an important approach for TCM studies. The development a of powerful, comprehensive TCM database and research has led to the treatment of various diseases like stroke, common cold, bronchitis, etc. (*Traditional Chinese Medicine: What You Need To Know | NCCIH*, n.d.)

2.5. Drug-likeness properties

Drug-likeness of compounds can be analyzed with SwissADME software. This software is freely available, and it allows the evaluation of pharmacokinetics, drug likeness, etc. Drug discovery has many developmental steps among which absorption, distribution, metabolism, and excretion (ADME) analysis is the most crucial step. (Daina et al., 2017a)

Compounds are shortlisted based on various rules. The most known is Lipinski's rule of five. The Rule-of-five explains the association between the pharmacokinetics and physicochemical parameters. Other filters include Ghose, Veber, Egan, and Muegge.

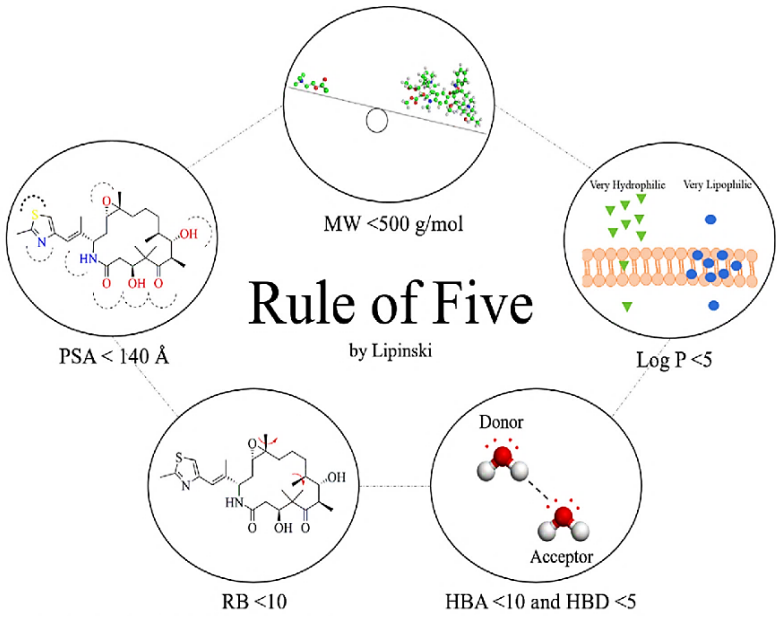


Figure 2.2. Image of the Rule of Five criteria: Molecular weight (MW), polar surface area (PSA), rotatable bonds (RB), hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), log P. (Chen et al., 2020)

2.6. Development of pharmacological network and pathways analysis

A crucial first step in the discovery and development of new drugs is the identification of novel targets. The pathogenesis of most diseases, including cancer and neurological

disorders, is complicated and involves numerous genetic and environmental aspects. It is difficult to identify an effective pharmacological target-drug combination that has a high probability of producing clinical success within the efficacy-toxicity range. (Harrold et al., 2013)

Therefore, Comparative Toxicogenomic Database (CTD), Therapeutic Target Database (TTD), and Drug Bank are among the most used databases that provide information regarding disease targets and bioactive compounds. Network construction is usually performed in STRING software, where the nodes represent the target proteins and edges are lines that represent the interactions between two or more nodes. This software provides information on protein-protein interactions and their biological and functional associations. (Szklarczyk et al., 2019). Cytoscape is a free online bioinformatics tool, used for visualizing molecular interactions. (Kohl et al., 2011)

2.7. Molecular docking

Molecular docking is a crucial tool in computer-assisted drug design (CADD) which can significantly reduce the research cost and enhance the efficacy to predict the binding affinity and interactive modes of the drug and its target. This tool allows ligand-protein docking which allows predicting the binding modes of the ligand with a three-dimensional protein structure. Molecular docking allows lead optimization, to produce huge compound libraries. These compound libraries function as a substrate which proposes the idea of how the ligand can inhibit the target. For docking, the protein structure is required to fit these compounds inside its various pockets. The stability of this interaction depends on the active site, binding energy, number of hydrogen bonds, root-mean-square deviation (RMSD) value an,d a few other factors (Morris & Lim-Wilby, 2008).

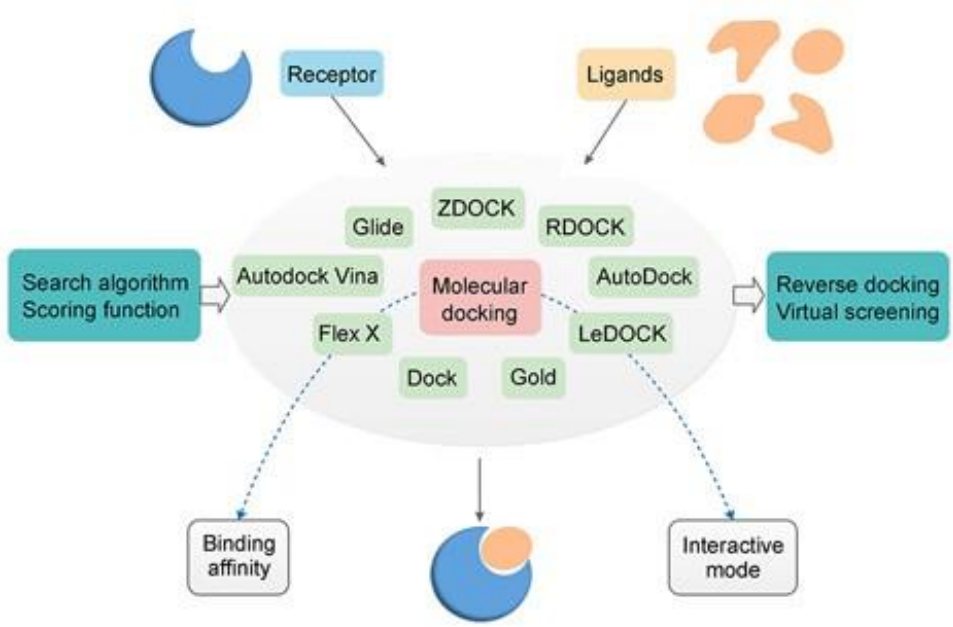


Figure 2.3. Molecular docking software (Fan et al., 2019)

Researchers found out that triptolide is a biological agent which interacts with targets of RA like RELA, MAPK8, CXCL8, STAT3, STAT1, TNF-α JNK, and c—JUN (Song et al., 2020). In another study, multiple protein targets and their agents have been reported. These targets mostly include interleukins and chemokines like TNF, IL-6, IL-2, IL-10, IL-15, IFN-γ, IL-17, CXCL10, CXCL8, etc. The agents or inhibitors are mostly monoclonal antibodies or their fragments (Huang et al., 2021).

2.8. MD Simulation

Molecular dynamics (MD) simulations provide several information regarding protein and ligand interactions, in addition to their stability of the dynamical structure. Such knowledge is crucial for determining the target's structure-function connection, the fundamentals of protein-ligand complexes, and for directing the drug discovery and design processes. As a result, MD simulations have been successfully utilized at every stage of contemporary drug research. (Liu et al., 2017)

A study provides MD simulations of TNF-α against two small molecules that function as its inhibitors. MD simulation at 100 ns was performed to examine the stability of complexes and the results concluded that these small molecules could be potential drug

inhibitors against TNF-α which is among the most common RA targets (Qaiser et al., 2020).

3. METHODOLOGY

3.1. In-vitro evaluation of therapeutic bioactive compounds in *Dodonaea viscosa*

3.1.1. Plant collection

Dodonaea viscosa was collected from Margalla Hills, Islamabad, Pakistan. Voucher specimens containing plant leaves, flowers, roots, and bark were deposited in the herbarium. After verification and authentication of the plant, accession number (00046453) was allotted by the Pakistan Museum of Natural History (PMNH), botanical sciences division, Shakarparian, Islamabad, Pakistan.

3.1.2. Plant material and extract preparation

The leaves of the plant were separated, cleaned, and washed under running tap water to remove dust. Then, they were shade dried at room temperature, for a week. The dried leaves were crushed and ground to a fine powder with the help of a mechanical blender. Later, the powder was stored in an air-tight container with proper labeling for further usage.

Extracts were prepared according to (Luque de Castro & García-Ayuso, 1998) protocol by the soxhlet extraction method with slight modifications. For Soxhlet extraction, 15g of dried plant powder was weighed and loaded in a thimble. Then, 200ml of ethanol (solvent) was filled in a round bottom flask independently. Everything was adjusted in the Soxhlet apparatus, and the temperature was set as required (50-60°C). Precaution is required while managing the Soxhlet apparatus, to avoid excess boiling of the solvent. The procedure required 2-3 days. After that liquid extract was obtained in the round bottom flask. Then, the filter paper was used to filter out the extract and was transferred to a clean flask, wrapped with aluminum foil. The extract was stored at 4°C in the refrigerator. Later, the filtrate was poured into clean petri plates and air-dried at room temperature. Dried extracts were then scratched from Petri plates. Crystallized dry

extracts were then stored in falcon tubes and placed in a refrigerator at 4°C for further use in phytochemical analysis.

3.1.3. Phytochemical screening of *Dodonaea viscosa*

Phytochemical analysis of leaf extract was performed by using standard methods.

1. **Alkaloids**

   Firstly, Hager's reagent was prepared by dissolving 1g of picric acid in 100ml of water. For the testing, 2ml of plant extract and a few drops of Hager's reagent were added. The presence of yellow precipitates indicates the presence of alkaloids.

2. **Phenols**

   Phenols were detected by using 1% $FeCl_3$. In 1ml of plant extract, a few drops of freshly prepared $FeCl_3$ were added. The bluish-black color indicates the presence of phenols.

3. **Anthraquinones**

   Reaction mixture contained 3ml extract, 3ml benzene solution and 5% NH3 (10%). The test tube was slightly mixed and observed. Pink or red color indicates its presence.

4. **Flavonoids**

   Flavonoids were detected using lead acetate. Ten percent of lead acetate solution was prepared in a falcon tube. 1ml of the extract was added to another test tube along with 1ml of 10% lead acetate. The presence of yellow precipitates indicates a positive result.

5. **Anthocyanins**

   In a test tube, 2ml extract, 2ml HCL (2N), and a few drops of NH3 were added. The red to bluish-violet color indicates the presence of anthocyanins.

6. **Tannins**

   This test is also named "Braymer's testing." 5% FeCl3 was prepared for the test. Then 2ml plant extract, 2ml water, and a few drops of 5% FeCl3 were added to a test tube and color was observed. The greenish to black color indicates the presence of tannins.

7. **Coumarins**

   First, a 10% NaOH solution was prepared. After that, 2ml extract and 3ml of NaOH (10%) were added to a test tube. Yellow coloration indicates its presence.

8. **Terpenoids**

   In a reaction tube, 2ml extract, 2ml ethanol, and 2ml chloroform were added, mixed, and then heated for 2mins. Then a few drops of H_2SO_4 were added. Deep red coloration indicated the presence of terpenoids.

9. **Sterols**

   In a test tube, 1ml of extract and a few drops of H_2SO_4 are added and mixed. The appearance of a red-colored ring at the bottom layer indicates the presence of sterols.

10. **Saponins**

    It is also known as the "Foam test." In this 5ml of the extract is added to 5ml of water and then slightly heated. The appearance of froth indicates the presence of saponins.

11. **Glycosides**

    The test tube mixture contains 2ml extract, 2ml chloroform, and 2ml of acetic acid (CH_3COOH). The violet-bluish color indicates the presence of glycosides.

12. **Cardiac glycosides**

    In a test tube, 2ml extract, 2ml acetic acid, 1ml H2SO4, and a few drops of FeCl3 were added to observe the presence of cardiac glycosides. The Violet color below the brown color indicates a positive result.

Table 3.3. List of phytochemicals with their procedures and appearance.

#	Metabolites	Procedures	Appearance
01	**Alkaloids**	2ml extract + a Few drops of Hager's reagent	Yellow precipitate
02	**Phenols**	1ml extract + Few drops $FeCl_3$	Bluish black color
03	**Anthraquinones**	3ml extract + 3ml Benzene + 5ml NH_3 (10%)	Violate or red color
04	**Flavonoids**	1ml extract + 1ml Pb $(C_2H_3O_2)_4$ (10%)	Yellow precipitate
05	**Anthocyanins**	2ml extract + 2ml HCl (2N) + NH_3	Bluish violet color
06	**Tannins**	2ml extract + 2ml H_2O + Few drops $FeCl_3$ (5%)	Greenish to black color
07	**Coumarins**	2ml extract + 3ml NaOH (10%)	Yellow color
08	**Terpenoids**	2ml extract + EtOH+2ml $CHCl_3$ + Δ (2 mint.) + 3 drops cH_2SO_4	Deep red color
09	**Sterols**	1ml extract + a Few drops of cH_2SO_4+ Shake + Stand	The red color appears at the lower layer
10	**Saponins**	5ml extract + 5ml H_2O + Heat	Froth appearance
11	**Glycosides**	2ml extract + 2ml $CHCl_3$ + 2ml CH_3 COOH	Bluish to green color
12	**Cardiac glycosides**	2ml extract + 2ml acetic acid + Few drops $FeCl_3$ + 1ml cH_2SO_4	Violate color below brown color

3.1.4. Total Phenolic content (TPC)

Total phenolic content is measured by using the Folin-Ciocalteu method as described by (Singleton et al., 1999) where the quantities of phenolic compounds are measured. These compounds are important for their redox properties and hence play a key role in antioxidant activities. The principle of the F-C assay includes the reduction of FC reagent in the presence of phenolic compounds. This causes the production of molybdenum-tungsten blue that is measured in a spectrophotometer at 620 nm.

Required chemicals: Gallic acid, 75% Na_2CO_3, Folin reagent, plant extract.

To determine the total phenolic content, dilutions of varying concentrations (25, 50, 75, 100 µg/ml) of gallic acid were prepared. Then 0.4ml FC reagent was added to 1ml of standard dilutions each. After waiting for 5mins, 4ml of 75% Na_2CO_3 was added to the tubes. Later, 4.6ml of distilled water was added to make it up to 10ml volume of the solution. The reaction tubes were incubated in dark for 2 hours. Finally, absorption was taken at 620nm in a microplate reader. A calibration curve for Gallic acid (standard) was used. Total phenolic contents were expressed as mg GAE/g dry extract. The procedure was performed in triplicate.

Calculations:

The standard curve was plotted (for gallic acid) which mentioned the equation and the R^2 value. The equation, Y= slope (X) + intercept was calculated for value 'X.' In this equation, 'Y' was the average absorbance of plant concentration and 'X' was the total phenolic content of extract, which had to be calculated. Standard deviation was calculated for the result.

3.1.5. Total Flavonoid content (TFC)

The protocol was followed from previously published work by (AL-Oraimi & Hossain, 2016c).

Required chemicals: Rutin, aluminum chloride, 1M potassium acetate

The method for this assay was the aluminum chloride colorimetric method. Firstly, 1mg of rutin was dissolved in 1ml methanol. Then various dilutions of rutin (25, 50, 75, 100 µg/ml) standard solution were prepared so that the total volume was up to 100 µl. Then, 100 µl of 10% AlCl3 was added. Along with 100 µl of 1M potassium acetate (KCH3COO). Then 2800 µl of distilled water was added to the reaction mix. The tubes were incubated for 30mins at room temperature (25°C). The absorbance was taken at 415 nm. A calibration curve for Rutin (standard) was used. Total flavonoid contents were expressed as mg RE/g dry extract. The procedure was performed in triplicate.

Calculations:

The standard curve was plotted (for Gallic acid) which mentioned the equation and the R^2 value. The equation, Y= slope (X) + intercept was calculated for value 'X.' In this equation, 'Y' was the average absorbance of plant concentration and 'X' was the total phenolic content of extract, which had to be calculated. Standard deviation was calculated for the result.

3.1.6 Antioxidant assays

The human immune system is responsible for the production of reactive oxygen species (ROS) which are destructive to the human body and worsen the disease symptoms if present in excess. Therefore, antioxidant assays are important to determine the potential of plant bioactive compounds to eliminate those reactive species, also known as antioxidant potential. Some assays performed to determine the antioxidant potential of *Dodonaea viscosa* are DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) and Ferric Reducing Antioxidant Power assay.

3.1.6.1. DPPH

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) is a dark-colored crystalline powder containing stable free radical molecules. This antioxidant assay determines free radical scavenging capacity. According to the key principle of this assay, free radicals form a stable complex at room temperature in the presence of an antioxidant molecule. DPPH,

which is originally violet-colored turns into a colorless solution once the stable complex is formed. A standard protocol (Blois, 1958) was followed in the following experiment with slight modifications.

Required chemicals: DPPH, methanol, ascorbic acid, plant extract

Firstly, 1mg of DPPH was weighed in dark and then dissolved in 25ml of methanol by using a vortex machine. The tube was covered in aluminum foil and incubated in the refrigerator at 4°C for 30mins before use. Then, ascorbic acid was prepared for which 25mg ascorbic acid was weighed and dissolved in 25ml of methanol. The tube was mixed well. Extract of *D.viscosa* was prepared in an Eppendorf by dissolving 1mg in 1ml of methanol and vortexed. In a 96-well plate, dilutions of various concentrations (10, 20, 30,40,50,60 µg/ml) were added in triplicates with a total volume of 200µl in each well. Ascorbic acid was used as standard. The plate was wrapped and incubated in dark for about one hour. Then absorption (OD) was taken in the microplate reader at 550nm. Percentage inhibition was calculated from the given formula.

Calculation:

Percentage inhibition = (1- Absorbance of sample/ Absorbance of control) × 100

3.1.6.2. FRAP

Ferric Reducing Antioxidant Power (FRAP) assay is an adaptable and economical tool for determining the antioxidant potential of the sample. According to the FRAP assay principle, ferric-tripyridyltriazine (Fe+3 -TPTZ) complex is reduced to ferrous-tripyridyltriazine (Fe=2-TPTZ) by the antioxidants of a sample at low pH. Standard protocol from (Benzie & Devaki, 2017) was used with slight modifications.

Required chemicals: 1% KFeCN, 10% TCA, 0.1% FeCl3, sodium phosphate buffer 0.2M maintained at 6.6 pH, ascorbic acid, sample solution.

First, dilutions of plant extract/ standard were made with varying concentrations (25, 50, 75, 100, 125, 150, 175 µg/ml) in falcon tubes. Each tube contained 1ml sample dilution, 2.5ml phosphate buffer, and 2.5ml of 1% KFeCN. The tubes were then incubated for 20mins at 50°C. After that, 2.5ml of 10% TCA was added and mixed well. Then

centrifugation was done at 3000rpm for 10mins. After centrifugation, 2.5ml of supernatant was obtained and added to a new falcon tube where 2.5ml of $FeCl_3$ was added. Absorbance was taken at 700nm in a spectrophotometer, and the results were analyzed. Ascorbic acid was used as a standard.

3.1.7. Anti-inflammatory assays

3.1.7.1. HRBC membrane stabilization assay

Human red blood cell membrane stabilization assay is performed to estimate the anti-inflammatory potential of our sample in vitro.

Required chemicals: 0.85% w/v normal saline, phosphate buffer solution (7.4 pH), whole blood, aspirin/diclofenac potassium, plant extract.

A standard method from (Parvin et al., 2015) was followed with slight modifications. 5ml of whole blood was drawn from a healthy volunteer. It was then centrifuged at 3000 rpm for 5 minutes. Washing with (0.85% w/v) normal saline was done thrice. Then remaining blood volume was measured and 1ml of that blood was for making 10% (v/v) blood suspension with normal saline solution. Firstly, various dilutions of different concentrations (100, 200, 300, 400 µg/ml) were made with extract/standard. In another falcon tube, 0.5ml of prepared sample dilution was added. Then 1ml normal saline, 0.5ml blood suspension, and 1ml of phosphate buffer solution (7.4 pH) were added in falcon, respectively. These tubes were incubated at 37°C for 30mins in a shaking water bath. After that, the tubes were centrifuged at 2500 rpm for 5mins, and absorbance was taken at 540nm. Phosphate buffer solution (PBS) was used as control whereas, diclofenac potassium was used as a standard drug.

Calculation:

Percentage inhibition = (1- Absorbance of sample/ Absorbance of control) × 100

3.1.7.2. Protein denaturation

Denaturation of proteins is known to be caused by inflammation in which secondary and tertiary structure of the protein is lost due to many external factors such as heat, strong

acids/ bases, or compounds. The principle of this assay is to evaluate the anti-inflammatory activity of plant extract where plant extract effectively inhibits protein denaturation. The protocol was obtained from (Umme & Hasan, 2019) with slight modifications.

Required chemicals: 1% BSA aqueous solution, PBS 0.2M, aspirin, plant extract.

For checking the protein denaturation, different concentrations (100, 200, 300, and 400 µg/ml) of extract/ standard were made in falcon tubes. Then 0.2ml of 1% BSA solution and 2.8ml of PBS (0.2M) were added to the same reaction tubes. The tubes were placed in a water bath at 37°C for 15mins. Then they were placed in an incubator at 70°C for 5mins. Tubes were then taken out and allowed to cool at room temperature for a few minutes. Finally, absorbance was taken at 620nm in the microplate reader. Aspirin was used as a standard drug.

Calculations:

Percentage inhibition = (1- Absorbance of sample/ Absorbance of control) × 100

3.2. In-silico evaluation of phytochemicals in *Dodonaea viscosa* and its potential targets against the treatment of Rheumatoid arthritis

3.2.1. GC-MS and Collection of Data

Data of plant phytochemicals were obtained by the Gas chromatography-mass spectrometry (GC-MS) technique of ethanol plant extract. For this, 1mg of dried plant extract was dissolved in 1ml of ethanol solvent and subjected to GC-MS analysis in QP-2020 SHIMADZU (Japan) system interfaced to SH-Rxi-5Sil mass spectrophotometer available in USPCASE, NUST. A standard procedure was used to generate a library of phytochemicals and then a further in-silico study was conducted.

3.2.2. Screening Lead Compounds Based on Drug-likeliness

After retrieving phytochemicals data of *Dodonaea viscosa* from the GCMS results, compounds were shortlisted based on different parameters in the following steps:

- Firstly, all the names of obtained phytochemicals were inserted on an excel sheet and a library was prepared.
- Then, the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was used to obtain details of these chemical compounds. PubChem ID, molecular weight, and canonical SMILES were obtained from this database and inserted in the previous excel file for each compound. All the duplicate compounds were eliminated from the list.
- Next, canonical SMILES of all phytochemicals were pasted in a free web tool, SwissADME (http://www.swissadme.ch/) which aids in the evaluation of pharmacokinetics and drug-likeliness of small molecules. It provides various parameters for a potent molecule to reach its target in the body in adequate quantities and remain in the bioactive form to be used as a medicinal compound. Absorption, distribution, metabolism, and excretion (ADME) testing is, therefore, significant in the drug development process (Daina et al., 2017b).
- Compounds' shortlisting criteria were based on physicochemical properties, water-solubility, pharmacokinetics (e.g., GI absorption, BBB permeation), medicinal chemistry, and drug-likeness including Lipinski's rule of five and a few others.
- After that compounds were analyzed to evaluate the cardiotoxicity via Human Ether-a-go-go-Related Gene (hERG) inhibition. Pred-hERG 4.2 (http://predherg.labmol.com.br/) is a free web computational tool for predicting cardiac toxicity since lethal cardiac arrhythmia is caused by the blockage of the hERG (K+) channels and therefore, plays a key role in drug development (Braga et al., 2015).
- Subsequently, these compounds are further shortlisted based on CYP450 inhibition from OCHEM (https://ochem.eu/home/show.do), which is an online chemical database with a modeling environment. CYP450 enzymes are membrane-bound hemoprotein that plays a significant role in the metabolism of drugs and

xenobiotics and maintains hemostasis. Moreover, drug-drug interactions are triggered by the induction or inhibition of these enzymes.

- Consequently, the list of these shortlisted compounds was finalized for further docking and can be our potent drug molecules.

3.2.3. Computational Target Prediction of Compounds

Potential biological targets of potent bioactive compounds were identified in the next step. RA targets for shortlisted compounds were obtained from databases like Comparative Toxicogenomic Database (CTD) and Therapeutic Target Database (TTD). For obtaining disease targets in CTD, the disease name (Rheumatoid arthritis) was entered in the search bar and checked. In the genes tab, a list of genes appeared for RA, and it was downloaded in CSV format which contained gene names and their symbols. Now, further information on these genes was obtained from UniProt (https://www.uniprot.org/) which is a freely accessible, high-quality information source of protein sequence and function. In this webpage, gene symbols were entered from an excel file to obtain UniProt IDs and protein names which were then entered into the excel sheet. The final excel file contained the list of all biologically active RA targets.

3.2.4. RA-target Network Construction via STRING

Network construction of RA targets was done using STRING software (https://string-db.org/). The multiple proteins option was selected from the left bar on the STRING homepage where names of obtained genes were pasted. The organism was selected as *Homo sapiens.* A network of many proteins was formed with various nodes and edges indicating the type and strength of interactions. The confidence level was set to 0.900 which indicated interaction score between proteins and disconnected nodes was hidden for clarity. STRING network was then exported to Cytoscape software for HUB gene analysis.

3.2.5. HUB Gene Analysis

For HUB gene analysis, Cytoscape 3.9.1 software was downloaded from https://cytoscape.org/ and extensions like Cytohubba and STRING were installed. Then, the STRING network of RA genes was exported to Cytoscape 3.9.1. Cytohubba was launched to calculate the node score of the top ten genes from the network. There are twelve different parameters in Cytohubba which show the top ten genes according to their ranks in each parameter. The node table option was selected from the bottom ribbon on the screen. The top ten genes of each category were noted in an excel sheet. Now, gene score was calculated from the list based on their ranking and score out of twelve. The top three genes with the highest score were selected as HUB genes for docking and further studies.

3.2.6. Structure Retrieval

After screening lead compounds and predicting the target genes of RA, the next crucial step is to retrieve the structures of both ligands (compounds) and targets (proteins). Compound structures were obtained from PubChem and downloaded in SDF format. Whereas protein structures were retrieved from Protein Data Bank (PDB) from https://www.rcsb.org/.

3.2.7. *In Silico* Molecular Docking

After structure retrieval of ligands and disease targets, we downloaded free PyRx virtual screening software (AUTO DOCK VINA) from the webserver https://pyrx.sourceforge.io/ to perform the docking of our shortlisted compounds. We also downloaded PyMOL software and Discovery studio software. These software's was freely available online and were used in protein visualization.

The active site is very crucial for accurate docking therefore multiple sources were used to find them. Therefore, literature on that structure was studied for finding the active site of the protein. It was then confirmed by PyMOL and Discovery studio visualizers. Amino acids of active site were noted for further usage. From the downloaded co-crystallized

structure of the protein, hetatoms including ligands, water molecules, and ions were removed. This purified protein was then saved in PDB format. Similarly, ligand/inhibitor was purified by removing protein chains, water molecules, and ions. Then the ligand was saved in PDB format as well.

In PyRx, the purified protein was uploaded and with the right-click, it was converted into a macromolecule. Then, in the Open Babble tab, ligands were uploaded, and their energies were minimized with the right click, they were then converted to PDBQT file format. 'Run Vina' button was pressed and then both ligand and protein pdbqt files were selected. 'Next' was pressed and then from the 'Molecules' tab, specific amino acids of the protein were selected. The grid was set and adjusted for selected amino acids. After that, the 'run vina' tab was pressed to initiate docking. It took around 5-10mins to dock complexes based on their protein sizes and ligands. Results were displayed for nine conformations were their binding energy scores and RMSD values. These results were saved in CSV format. These docked structures were visualized in PyMOL and Discovery studio. Then, their docked complexes with the best binding energies were saved in PDB format for further analysis.

3.2.8. MD Simulations of docked complexes

Molecular dynamics simulations were conducted using the GROMACS simulation tool (GROMACS 2020.4). A 100 ns MD simulation of two complexes, (a) Protein-compound complex and (b) Protein-inhibitor complex, was performed in water using the CHARMM36m forcefield for the protein and the Charmm General force field for the organic ligand. Trajectory and energy files were recorded every 10 ps.

TIP3P water molecules were used to solvate the system in a shortened octahedral box. To effectively comply with the minimum image convention, the protein was centered within 1 nm of the box edge of the simulation box.

To avoid any steric collisions, minimization was performed using the steepest descent method for 5000 steps, achieving convergence within the maximum force of 1000 (KJ mol-1 nm-1). To achieve a properly converged system for the production run, the system

was equilibrated at NVT and NPT ensembles for 100ps (50,000 steps) and 1000ps (1,000,000 steps), respectively, utilizing time steps of 0.2 and 0.1 fs at 300K.

Weak coupling velocity-rescaling (modified Berendsen thermostat) and Parrinello-Rahman techniques were used to produce a simulated production run at a constant temperature of 310.15 K and a constant pressure of 1 atm or bar (using NPT ensemble). τ T = 0.1 ps and P = 2.0 ps were chosen as the relaxation times. Using the Linear Constraint Solver (lincs) algorithm with a 2-fs time step, all hydrogen atom-involving bond lengths were kept rigid at ideal bond lengths. The Verlet approach was applied to calculate the non-bonded interactions. Periodic Boundary Conditions (PBC) were employed in all x, y, and z directions. In each time step, interactions within a short-range cut-off of 1.2 nm were calculated. The electrostatic interactions and forces for a homogenous medium outside the long-range cut-off were calculated using Particle Mesh Ewald (PME). For both systems, the production was run for 100ns.

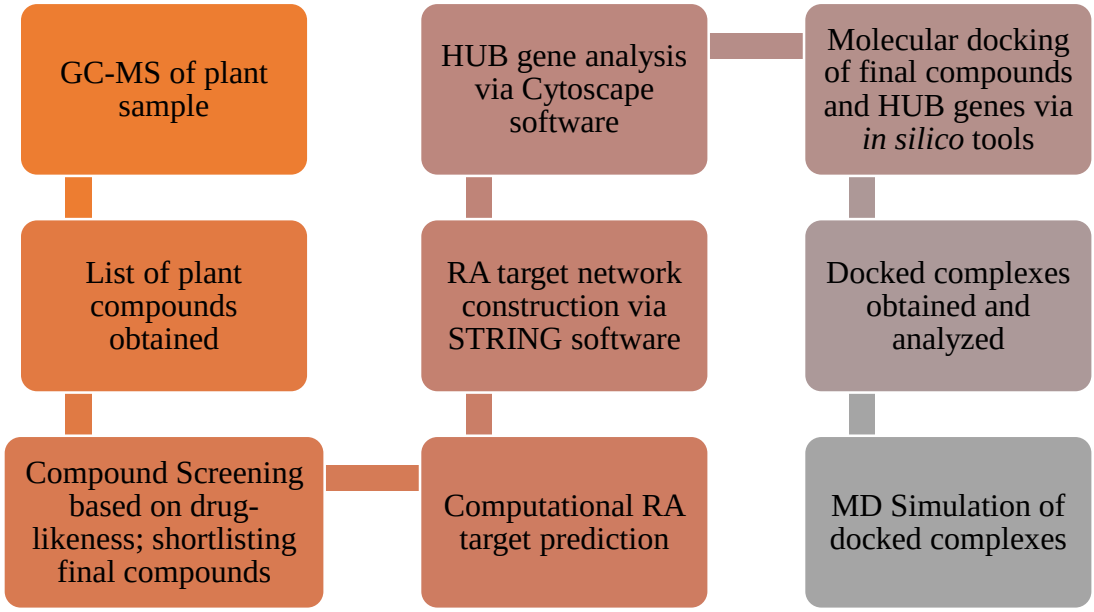


Figure 3.1. Schematic illustration of CADD molecular drug designing.

4. RESULTS

4.1. Plant Collection

Dodonaea viscosa used in this study was allotted an accession number 00046453. Fresh leaves of the plant were collected from Margalla Hills, Islamabad, Pakistan.

4.2. Management of Plant Extract

After obtaining ethanolic plant extract by the soxhlet method, the extract was filtered was kept at 4°C in an air-tight bottle wrapped with a foil. The extract had various physical properties including the ark green color and pungent smell. The extract was air-dried in a petri plate for over a week and then the remaining dried extract was scratched from the plate. A blackish powder with a dry crystalline structure was obtained. It was finely ground with the help of mortar and pastel and then stored in a wrapped falcon at 4°C for further experimental use.

Figure 4.1. Dried leaves of *Dodonaea viscosa* (Left). Soxhlet extraction of plant phytochemicals (Middle). Prepared ethanolic extract of *Dodonaea viscosa* (Right).

4.3. Phytochemical Screening of Plant Extract

Ethanolic extract of *Dodonaea viscosa* was used for phytochemical screening according to the methods previously explained in chapter 03. Below are the results of the phytochemical analysis.

4.3.1. Phytochemical Testing

Table 4.1. *Dodonaea viscosa* was evaluated for the presence of various phytochemicals.

S. no.	Test	*D. viscosa*	Appearance
1	Alkaloid	+	Yellow precipitate
2	Phenols	++	Bluish black color
3	Anthraquinones	-	Violate or red color
4	Flavonoids	+	Yellow precipitate
5	Anthocyanins	-	Red to Bluish color
6	Tannins	+	Greenish to black color
7	Coumarins	++	Yellow color
8	Terpenoids	++	Deep red color
9	Sterols	++	Red color appears at the lower layer
10	Saponins	+	Froth appearance
11	Glycosides	-	Blue to Green color
12	Cardiac glycosides	+	Violate color below the Brown color
13	Steroids	++	Reddish-brown color

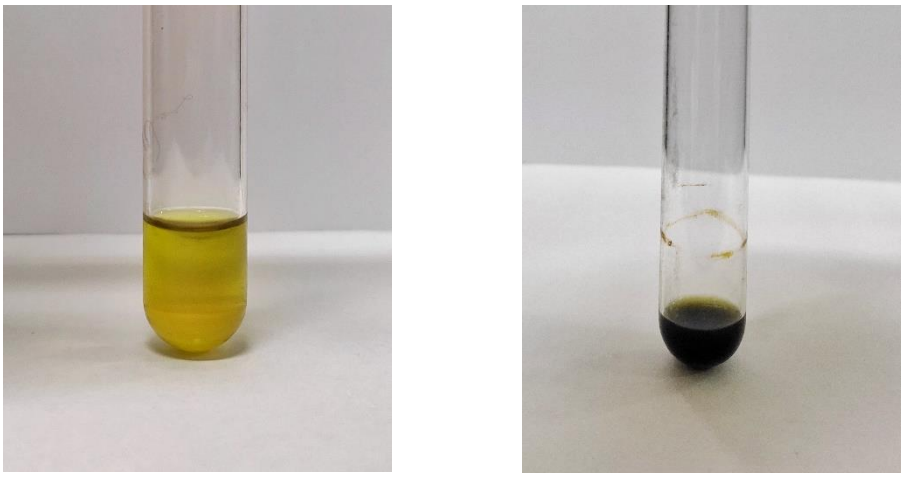


Figure 4.2. Positive result of both alkaloids (left) and phenols (right) in ethanol extract.

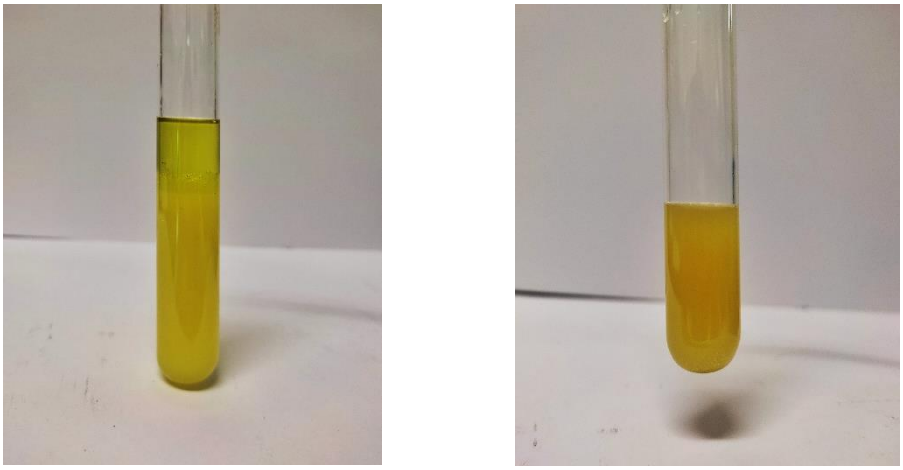


Figure 4.3. Negative result of both anthraquinones (left) and anthocyanins (right) in ethanol extract.

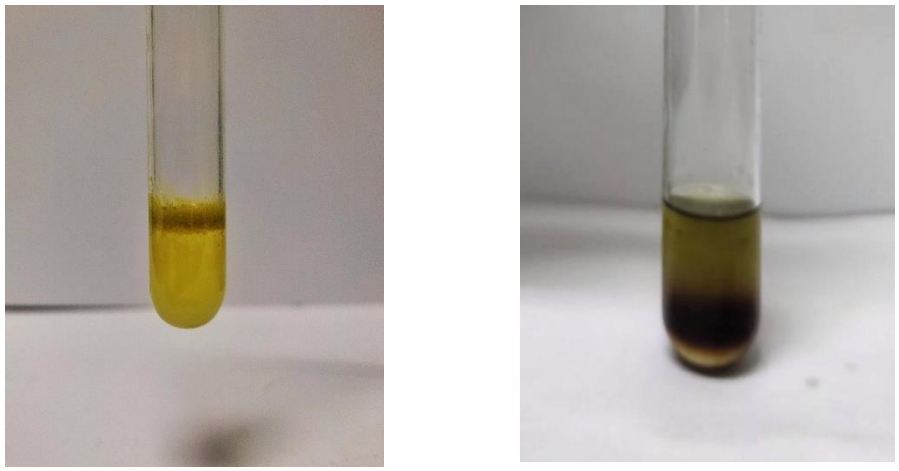


Figure 4.4. Positive result of both flavonoids (left) and cardiac glycosides (right) in ethanol extract.

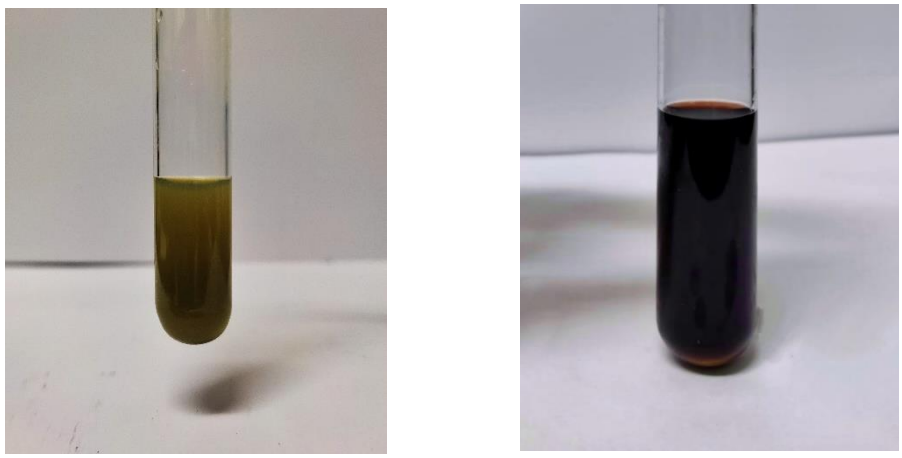
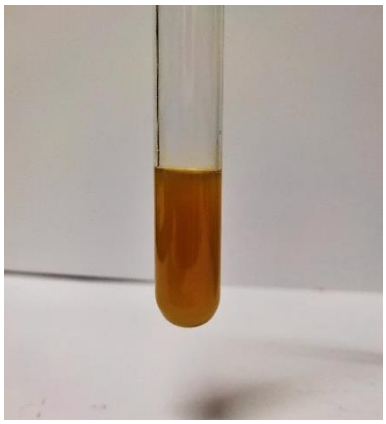


Figure 4.5. Positive result of both tannins (left) and terpenoids (right) in ethanol extract.



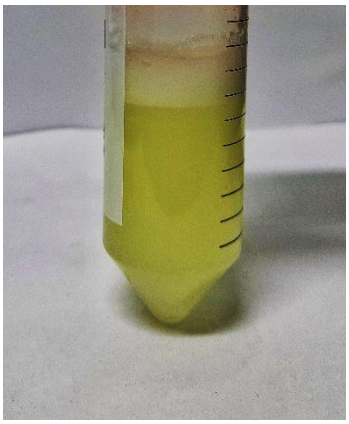
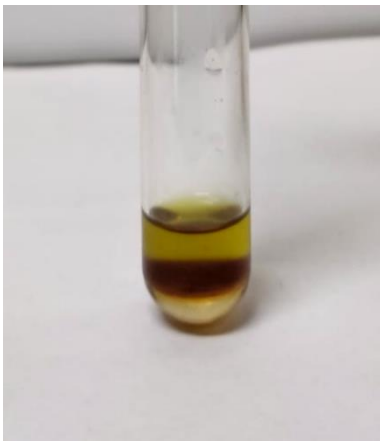


Figure 4.6. Positive result of both coumarins (left) and saponins (right) in ethanol extract.



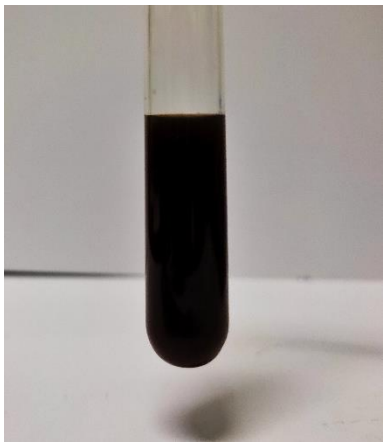


Figure 4.7. Positive result of sterols (left) and steroids (right) in ethanol extract.

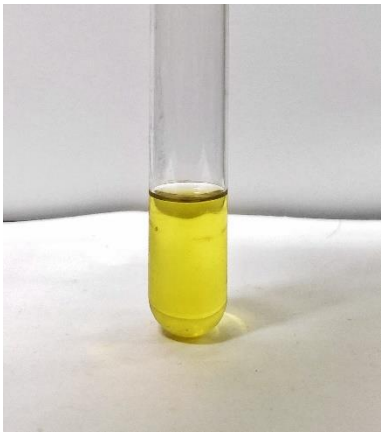


Figure 4.8. Negative result of glycosides in ethanol extract of *Dodonaea viscosa*.

4.4. Antioxidant Assays

4.4.1. DPPH Assay

The results of DPPH (2, 2-diphenyl-1-picrylhydrazyl) demonstrate the antioxidant activity of *Dodonaea viscosa*. They were evaluated by using the standard formula:

Percentage inhibition = (1- Absorbance of sample/ Absorbance of control) × 100

The graphical results were obtained by linear regression which illustrates an increase in % inhibition with increasing concentration of extract. Ascorbic acid was taken as a positive control. The ethanolic plant extract has significantly higher antioxidant activity with p-value ≤ 0.05 as compared to ascorbic acid, which means that plant extract has a higher percentage to inhibit free radicals as compared to ascorbic acid. Statistical analysis was performed by using Origin software.

DV (ethanolic extract); $R^2 = 0.930$, P-value 0.0018

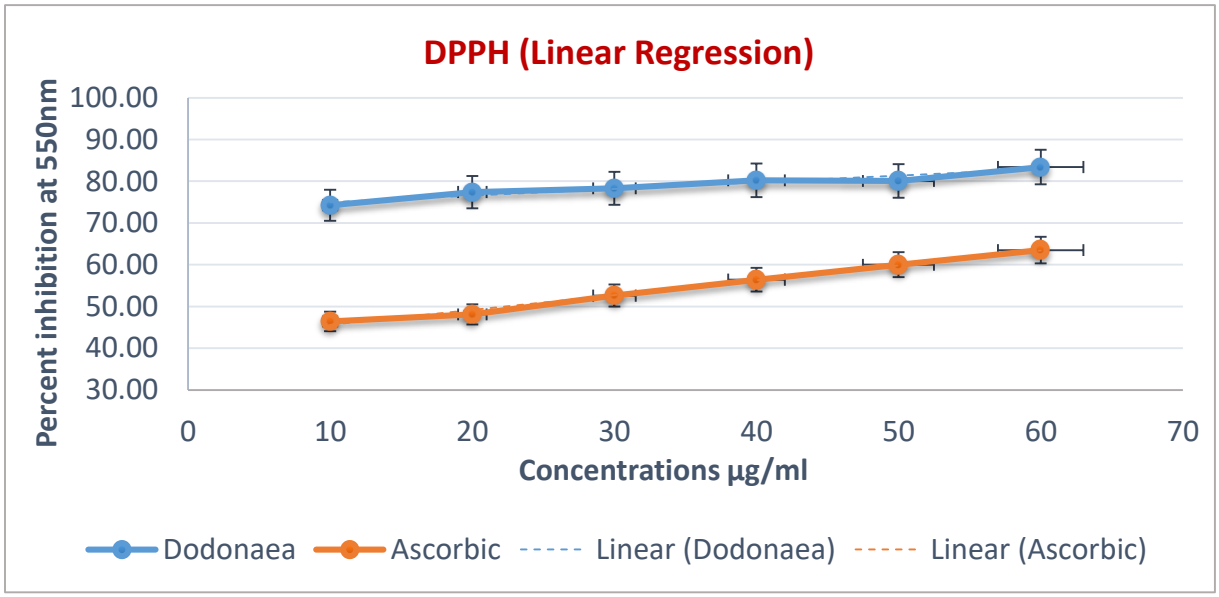


Figure 4.9. DPPH activity of ethanolic extract of *Dodonaea viscosa* leaves.

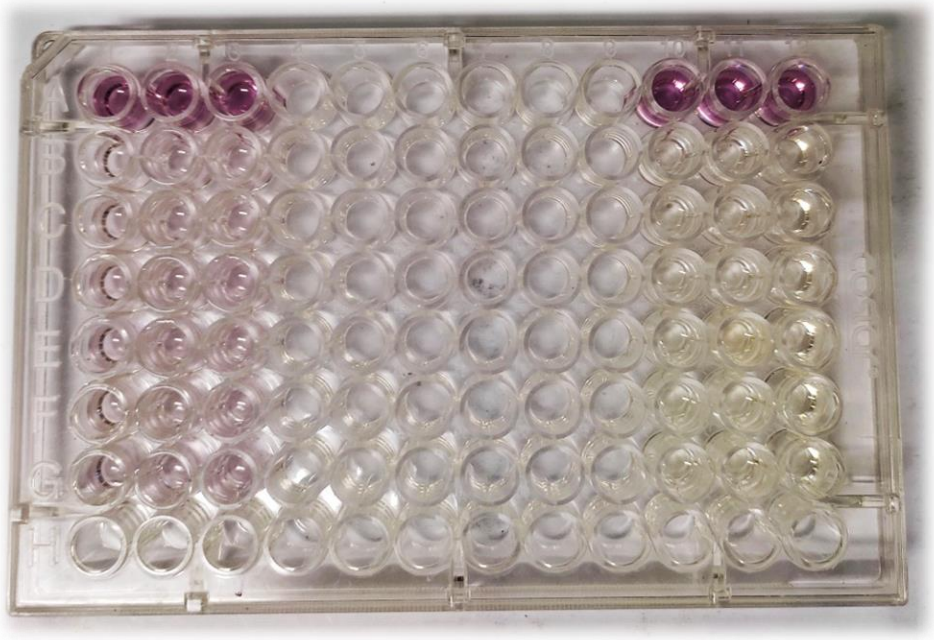


Figure 4.10. DPPH results of ascorbic acid and ethanol extract of the plant.

4.4.2. FRAP Assay

FRAP (Ferric Reducing Antioxidant Power Assay) was used to evaluate the ability of *Dodonaea viscosa* to reduce ferric ions to ferrous ions by its antioxidant potential. The results of FRAP were calculated by taking absorption at 700nm. The results in graphs were obtained by Linear Regression and the antioxidant potential of ethanolic plant extract is concentration dependent. Ascorbic acid used as a standard in this assay has shown greater absorbance at 700nm, whereas the ferric reduction potential of *D. viscosa* is slightly lower with a p-value ≤ 0.05. Statistical analysis was performed using Origin software.

DV (ethanolic extract); $R^2 = 0.984$, P-value 1.20E-05

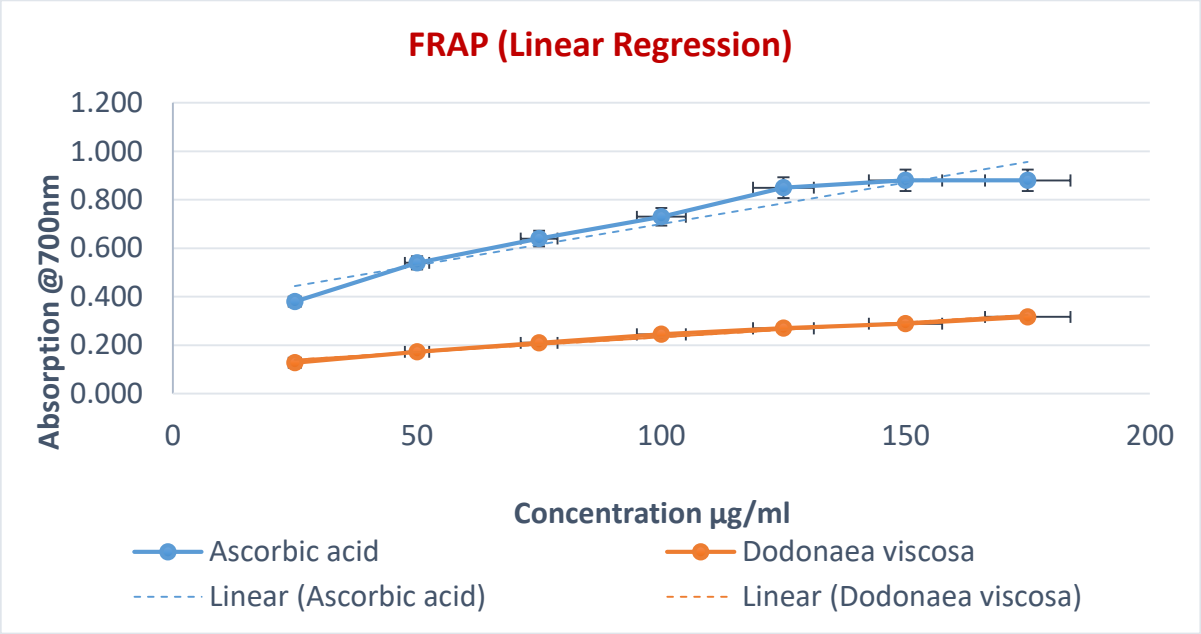


Figure 4.11. FRAP activity of *Dodonaea viscosa*.

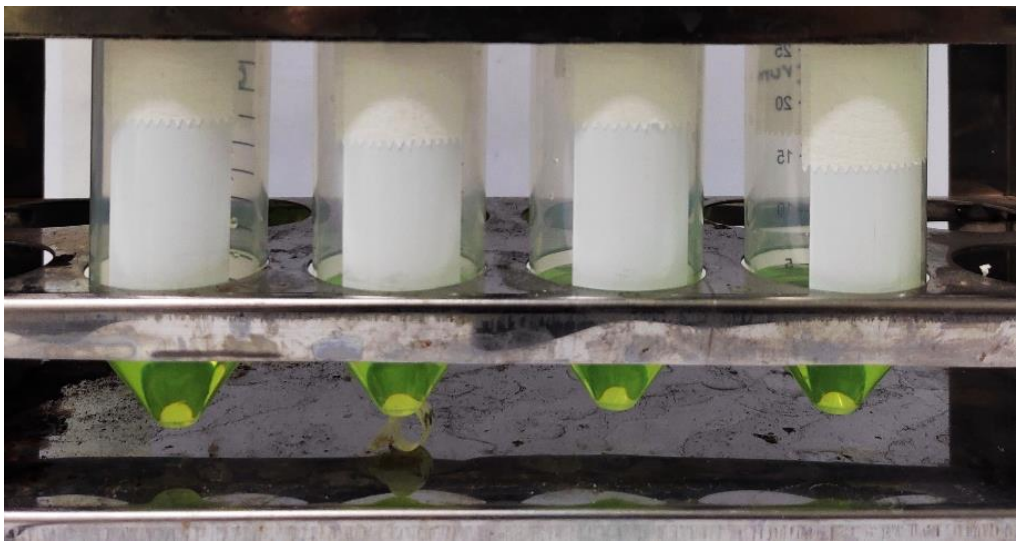


Figure 4.12. Results of FRAP assay

4.5. Anti-Inflammatory Assays

4.5.1. Protein Denaturation

The protein denaturation assay demonstrates the anti-inflammatory potential of *Dodonaea viscosa*. The results were evaluated by using the following formula:

Percentage inhibition = (1- Absorbance of sample/ Absorbance of control) × 100

The results were obtained by linear regression which illustrates an increase in % inhibition with increasing concentration of extract at 620nm. Diclofenac potassium was taken as a positive control. The ethanolic plant extract has significantly higher anti-inflammatory activity i.e., p-value ≤ 0.05, with respect to the standard. Statistical analysis was performed by using Origin software.

DV (ethanolic extract); $R^2 = 0.9624$, P-value 0.01897

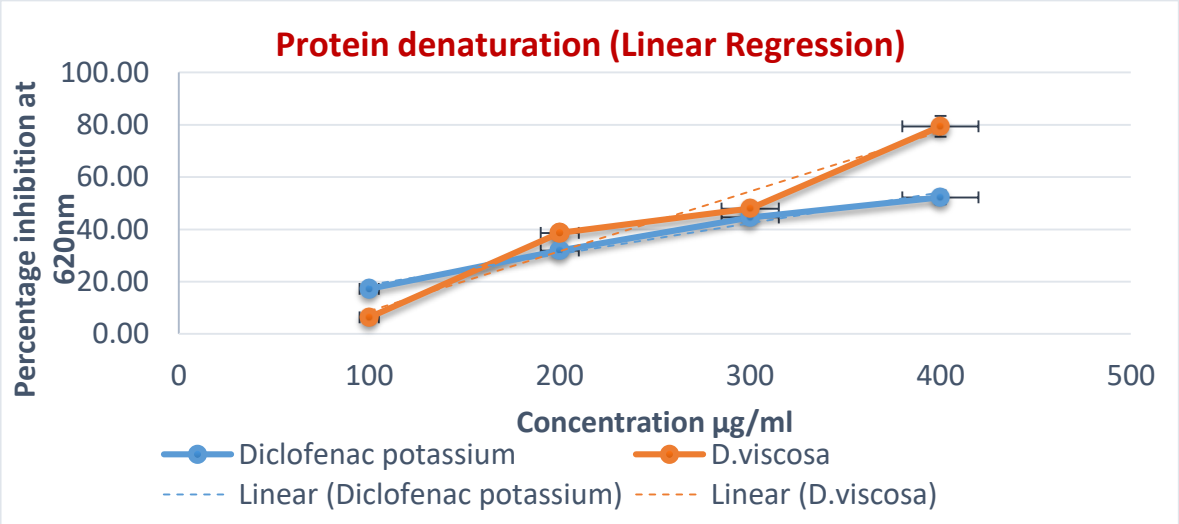


Figure 4.13. Protein denaturation activity of *Dodonaea viscosa*.

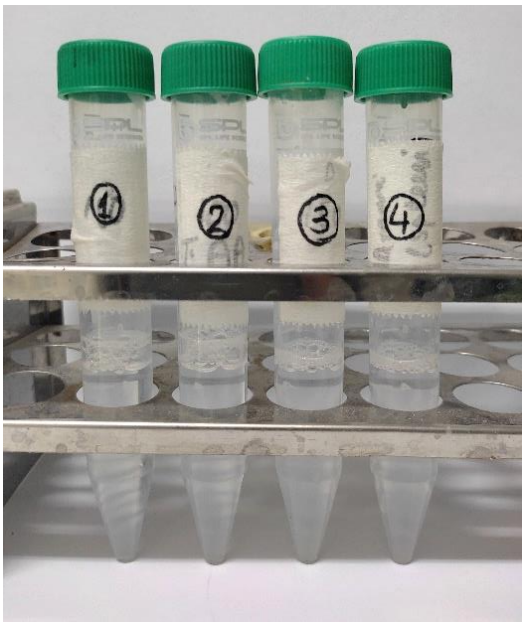


Figure 4.14. Evaluation of protein denaturation activity.

4.5.2. HRBC Membrane Stabilization Assay

The results of the HRBC membrane stabilization assay demonstrate the anti-inflammatory potential of *Dodonaea viscosa*. The results were evaluated by using the standard formula:

Percentage inhibition = (1- Absorbance of sample/ Absorbance of control) × 100

The graphical results were obtained by linear regression which illustrates an increase in % inhibition with increasing concentration of extract at 620nm. Diclofenac potassium was taken as a positive control. Ethanolic plant extract have significantly higher anti-inflammatory activity i.e., p-value ≤ 0.05 as compared to aspirin. Statistical analysis was performed by using Origin software.

DV (ethanolic extract); $R^2 = 0.9788$, P-value 0.00132

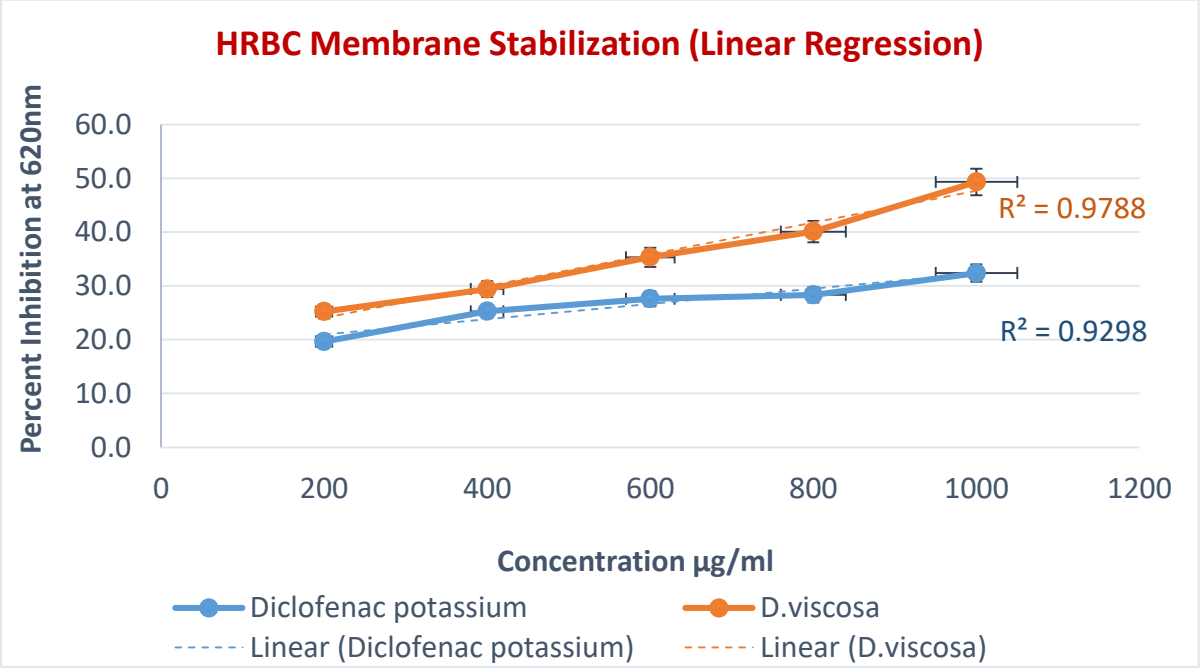


Figure 4.15. Percent inhibition of HRBC membrane stabilization of ethanolic *D. viscosa* extract.

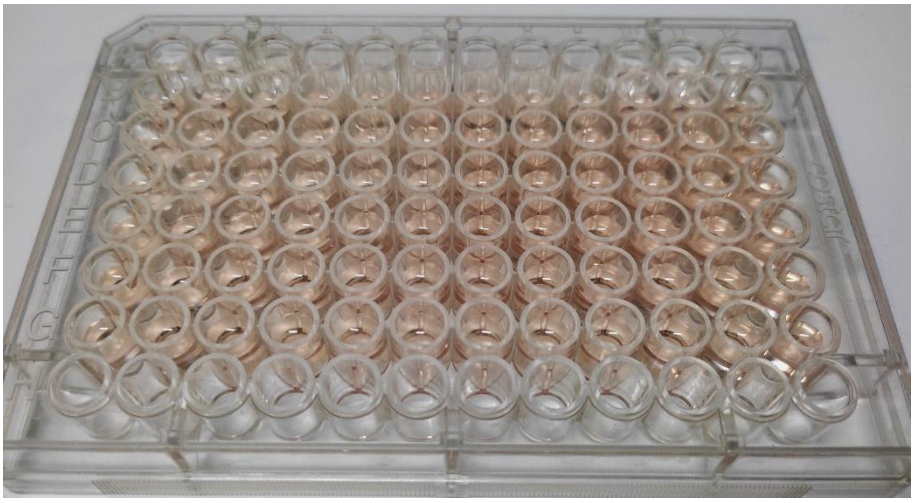


Figure 4.16. Evaluation of HRBC membrane stabilization activity for aspirin (standard) and *Dodonaea viscosa* (plant extract).

4.6. Total Phenolic Content (TPC)

The quantitative assessment of total phenolic content was conducted using the Folin-Ciocalteu reagent. TPC is expressed as mg gallic acid equivalent per gram of dry extract i.e., mg GAE/g dry extract. The results in the graph below were obtained by linear regression. The results in the table below were calculated from the linear regression equation. The results depict the presence of significant amounts of phenols in the ethanolic extract of *D. viscosa* (494.44 ± 0.004 mg GAE/g dry extract).

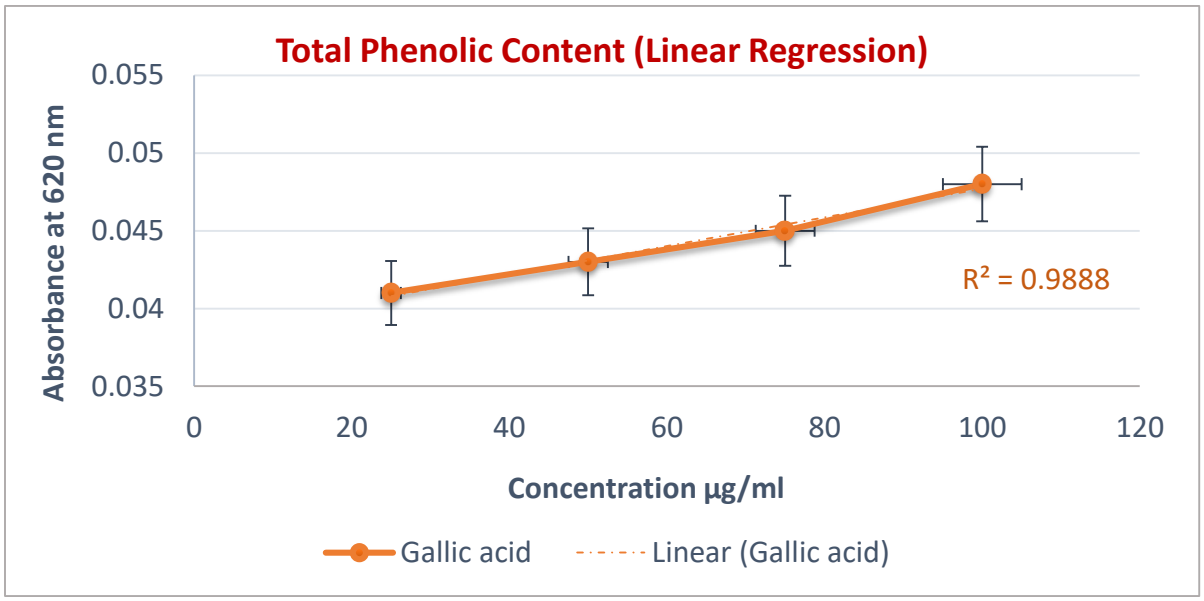


Figure 4.17. Total phenolic content for standard Gallic acid.

Extract	mg GAE/g dry extract
DV ethanolic extract	494.44 ± 0.0041

***Values (mean ± SD)**

Figure 4.18. Results of the total phenolic content of ethanol extract of *D. viscosa*.

4.7. Total Flavonoid Content (TFC)

The aluminum chloride colorimetric assay was used to calculate the total flavonoid content of ethanolic extract of *Dodonaea viscosa*, where rutin was used as a standard. TFC is expressed as mg rutin equivalent per gram of dry extract i.e., mg RE/g dry extract. Results in the graph below were obtained by linear regression. Results in the table below were calculated from the linear regression equation. The results depict the presence of significant amounts of flavonoids in the ethanolic extract of *D. viscosa* (883 ± 0.003mg RE/g dry extract).

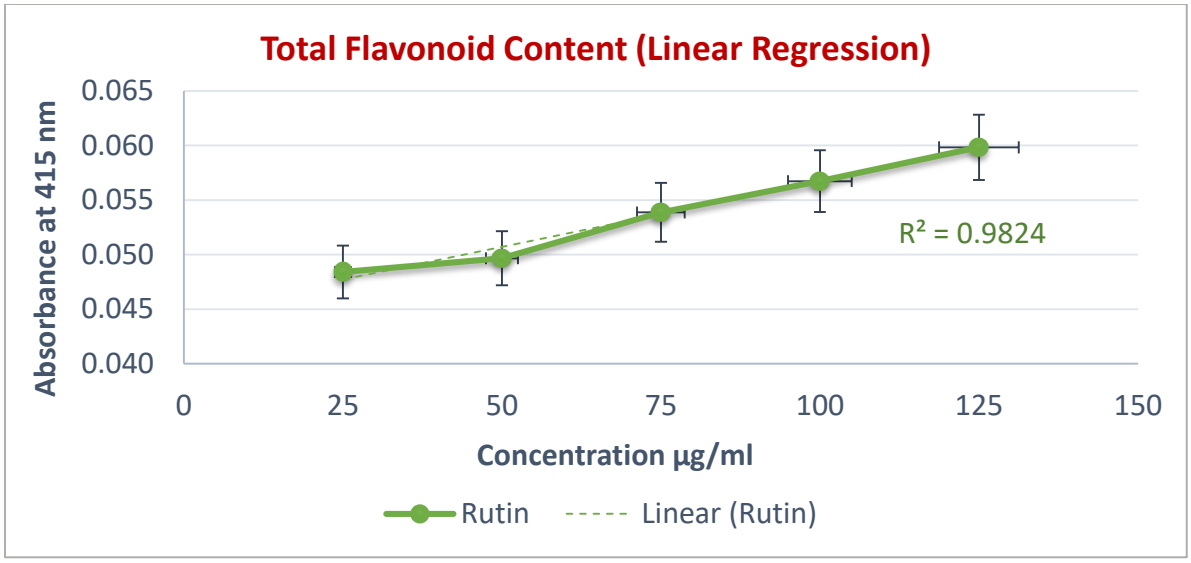


Figure 4.19. Total flavonoid content for standard Rutin.

Extract	mg RE/g dry extract
DV ethanolic extract	883 ± 0.003

***Values (mean ± SD)**

Figure 4.20. Results of the total flavonoid content of ethanol extract of *D. viscosa*.

4.8. Evaluation of phytochemical components and their *in-silico* analysis of *Dodonaea viscosa*

Dodonaea viscosa was the plant of interest in this study and its bioactive compounds were required to proceed with *in silico* analysis. Therefore, the compounds of its leaves were obtained by dissolving 1mg of dry plant extract in 1ml of ethanol for Gas chromatography-mass spectrometry (GC-MS) analysis. This provided a list of polar compounds present in the extract.

After GC -MS, a total of 480 compound was obtained. Then they were shortlisted from SwissADME, a free online tool based on Lipinski's rule of five. Other filters considered included Ghose, Veber, Egan, and Muegge. Criteria for topological polar surface area (TPSA) values were also checked. Due to these criteria 144 compounds were finalized. After expanding the criteria to molecular weight, blood-brain barrier (BBB) permeability, gastrointestinal (GI) absorption, water solubility, and medicinal chemistry violations, the final shortlisted compounds were 18. Then, these compounds were checked for cardiotoxicity from Pred-hERG and CYP 450 inhibition.

After shortlisting, 9 final compounds obtained were **(1)** 7-(2-Fluorophenyl)-4H,7H-[1,2,4]triazolo[1,5-a]pyrimidine-5-carboxylic acid **(2)** 4-Piperidinecarboxamide, 1-[2-(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]- **(3)** 2-(4-Hydroxypyrimidin-2-ylsulfanyl)-N-(5-methylisoxazol-3-yl)acetamide **(4)** Acetamide, N-methyl-2-[(5-methyl-1,3,4-thiadiazol-2-yl)thio]-N-phenyl **(5)** 5-(p-Acetylaminophenylsulfonyl)dihydro-1,3,5-dioxazine (6) 4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl)benzoic acid **(7)** 3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-hydroxy-2H-isoindol-1-one **(8)** 4-Acetyl-5-(furan-2-yl)-3-hydroxy-1-(pyridin-3-yl)-5H-pyrrol-2-one **(9)** 5,6,7-Trimethyl-[1,2,4]triazolo[1,5-a]pyrimidin-2-ylsulfanyl)-acetic acid.

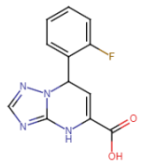
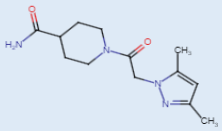
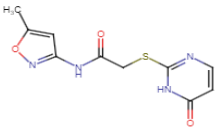
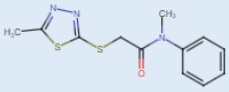
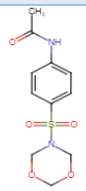
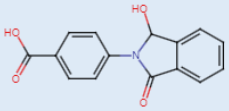
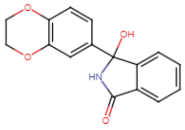
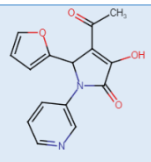
Table 4.2. Inclusion/ exclusion criteria for shortlisting of the compounds.

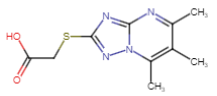
Name	Criteria
Lipinski and other violations	Zero violations
Molecular weight (MW)	150-500
TPSA	20-130
GI absorption	High
BBB permeability	No
Medicinal chemistry violations	Zero violations
Water solubility	Soluble/ very soluble
Cardiotoxicity (hERG prediction)	No
CYP450 inhibition	Non-inhibitor

Table 4.3. Parameters for compound shortlisting based on drug-likeness by Lipinski, and other filters. (HBD, H-bond donor; HBA, H-bond acceptor; #RB, number of rotatable bonds).

Molecule	MW	TPSA Å²	Heteroatom	HBD	HBA	#RB
1	260.22	80.04	19	2	5	2
2	264.32	81.22	19	1	3	4
3	266.28	126.18	18	2	5	5
4	279.38	99.63	18	0	3	5
5	286.3	93.32	19	1	6	4
6	269.25	77.84	20	2	4	2
7	283.28	67.79	21	2	4	1
8	284.27	83.64	21	1	5	3
9	252.29	105.68	17	1	5	3

Table 4.4. Details of nine shortlisted bioactive compounds.

S#	Compound Name	MW g/mol	PubChem ID	Structure
1	7-(2-Fluorophenyl)-4H,7H-[1,2,4]triazolo[1,5-a]pyrimidine-5-carboxylic acid	260.22	135645702	
2	4-Piperidinecarboxamide, 1-[2-(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-	264.32	951128	
3	2-(4-Hydroxypyrimidin-2-ylsulfanyl)-N-(5-methylisoxazol-3-yl)acetamide	266.28	753333	
4	Acetamide, N-methyl-2-[(5-methyl-1,3,4-thiadiazol-2-yl)thio]-N-phenyl	279.4	555687	
5	5-(p-Acetylaminophenylsulfonyl)dihydro-1,3,5-dioxazine	286.31	536713	
6	4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl)benzoic acid	269.25	18873897	
7	3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-hydroxy-2H-isoindol-1-one	283.28	91723927	
8	4-Acetyl-5-(furan-2-yl)-3-hydroxy-1-(pyridin-3-yl)-5H-pyrrol-2-one	284.27	91710979	

9	5,6,7-Trimethyl-[1,2,4]triazolo[1,5-a]pyrimidin-2-ylsulfanyl)-acetic acid	252.3	672041	

4.9. Retrieved RA targets

Targets of rheumatoid arthritis were searched in CTD database and from literature. Total 200 targets were retrieved. They were then verified from UniProt database for "*Homo sapiens*." An excel file having the list of targets i.e., protein names along with its UniProt ID and gene symbol was prepared. The final excel file contained the list of all biologically active RA targets.

4.10. Target Network Construction via STRING

The retrieved targets were imported to STRING software for network construction. After adjusting the required settings, a network of interconnected 193 nodes and 328 edges was formed. The network had PPI enrichment p-value: < 1.0e-16 which indicates that the network had significantly more protein-protein interactions. This network was then transported to Cytoscape software for HUB gene analysis.

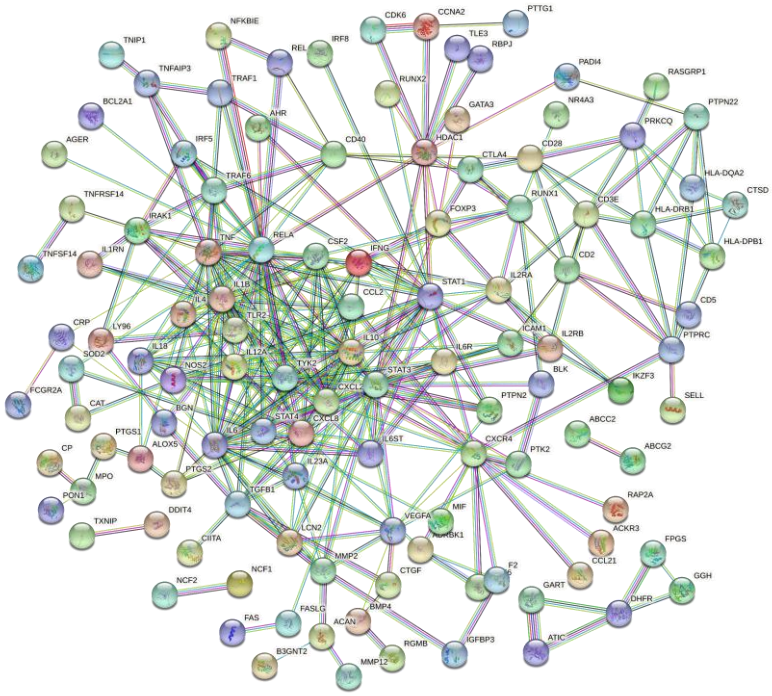


Figure 4.21. STRING network of 200 RA targets.

4.11. HUB Gene Analysis via Cytoscape Software

The STRING network imported to Cytoscape, opened Cytohubba extension of the software. The top 10 nodes ranked by various parameters were calculated and recorded in an excel file as shown in the **Figure 4.5.** below. Node scores were calculated for each gene and those three with the highest score were shortlisted for further analysis. As shown in the table, TNF had the highest score i.e., 10 out of 11 parameters were fulfilled. Whereas STAT3 and IL-6 were second and/ or third, based on their same scores.

Table 4.5. Top ten nodes ranked by different parameters in Cytoscape.

S#	MCC	MNC	DMNC	Degree	EPC	Eccentricity	BottleNeck	Closeness	Radiality	Betweenness	Stress
1	IL6	STAT3	CSF2	STAT3	STAT3	IL4	STAT3	STAT3	STAT3	STAT3	STAT3
2	IL1B	RELA	CCL2	RELA	IL6	CXCL2	CXCR4	RELA	RELA	CXCR4	CXCR4
3	IL18	IL6	MMP2	IL6	RELA	TYK2	RELA	IL6	IL6	RELA	RELA
4	CCL2	IL10	LCN2	IL10	IL10	TNF	TNF	STAT1	STAT1	HDAC1	TNF
5	IL10	TNF	IL18	TNF	TNF	IL12A	HDAC1	TNF	TNF	STAT1	STAT1
6	CSF2	IL1B	TGFB1	STAT1	IL1B	CCL2	CD40	IL10	IL10	TNF	ALOX5
7	CXCL8	STAT1	CXCL2	IL1B	STAT1	STAT4	IL6	IL1B	CXCL8	ALOX5	HDAC1
8	IL4	IL12A	IL12A	CXCR4	IL12A	ALOX5	CD28	CXCR4	CXCR4	MMP2	RUNX1
9	TNF	CXCL8	CXCL8	IL12A	IL4	IL1B	CXCL2	CXCL8	IFNG	PTPRC	IL6
10	IL12A	IL4	IL4	CXCL8	CXCL8	IL18	PTPRC	IFNG	IL1B	IL10	IL10

HUB Genes	Score
TNF	10
STAT3	8
IL6	8

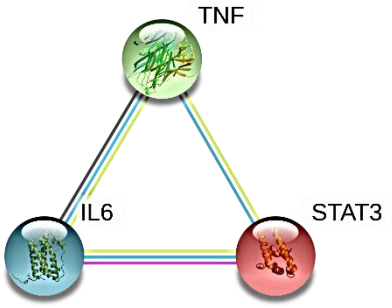
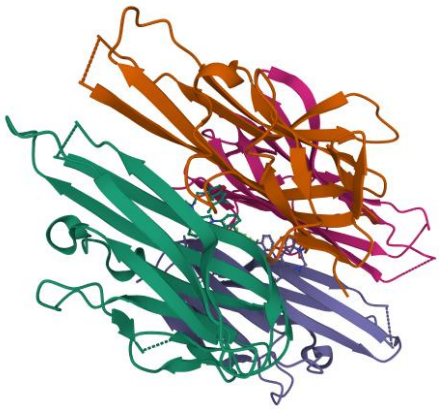
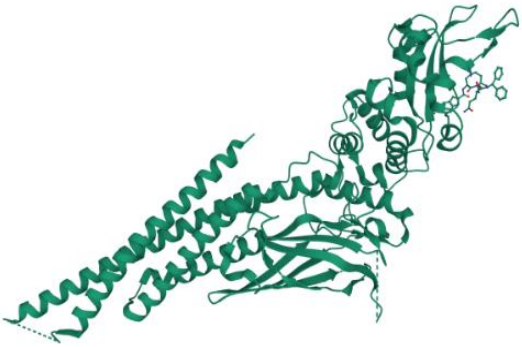


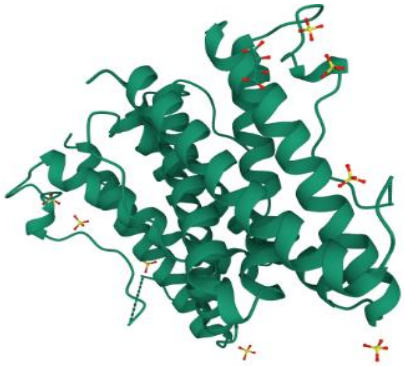
Figure 4.22. Top three genes based on HUB gene analysis.



Gene names: TNF, TNFA
PDB: 2AZ5
X-ray diffraction
2.1Å resolution
Chains: A, B, C and D
Length: 148 amino acids



Gene name: STAT3
PBD: 6NJS
X-ray diffraction
2.7Å resolution
Chain: A
Length: 562 amino acids



Gene names: IL6, IFNB2
PDB: 1ALU
X-ray diffraction
1.9Å resolution
Chain: A
Length: 186 amino acids

Figure 4.23. Details of the selected HUB genes.

4.12. Molecular Docking Analysis

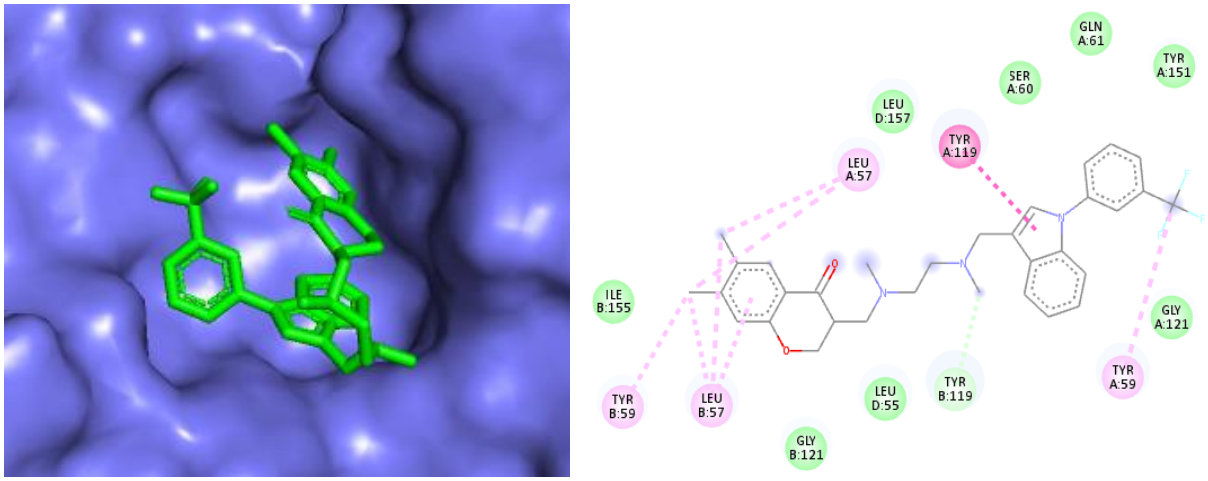
The top three genes obtained from HUB gene analysis participate in various pathways of RA including NF-kappa B signaling pathway, MAPK signaling pathway and JAK/STAT signaling pathway. Molecular docking was performed on PyRx (AutoDock Vina) software. The PDB structures of protein targets i.e., TNF, STAT3 and IL-6 were obtained and prepared for docking. All nine ligands/ plant compounds were also prepared for docking. Now, three target genes and nine plant ligands were imported to PyRx for molecular docking verification.

The original ligands of retrieved PDB structures were re-docked to verify the docking results. The results of molecular docking are shown in the **Table 4.6.** with their binding energy scores against each target.

Table 4.6. Molecular docking scores of TNF-α, STAT3 and IL-6.

Name	TNF-alpha binding energy	STAT3 binding energy	IL-6 binding energy
INHIBITOR	-8.6	-7.2	-3.7
Compound 1	-8	-6.5	-6.1
Compound 2	-6.9	-6.3	-5.7
Compound 3	-6.7	-6.1	-5.6
Compound 4	-6.6	-6.1	-5.6
Compound 5	-6.9	-6.1	-5.7
Compound 6	-8.2	-6.5	-6.3
Compound 7	-8	-7.1	-7
Compound 8	-7	-6.3	-5.5
Compound 9	-6.6	-5.5	-5.8

(a)



(b)

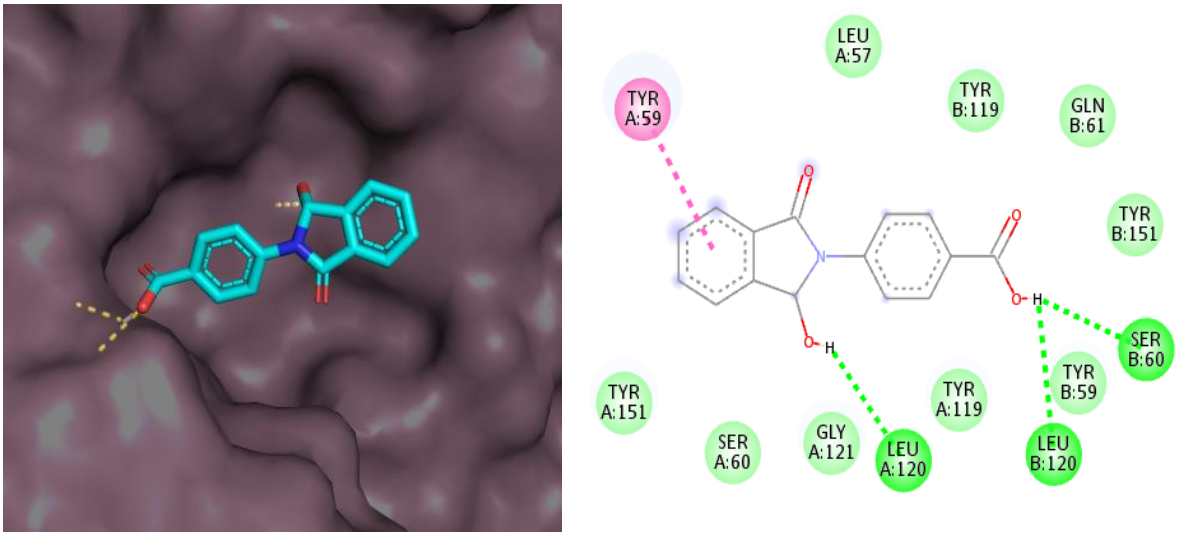
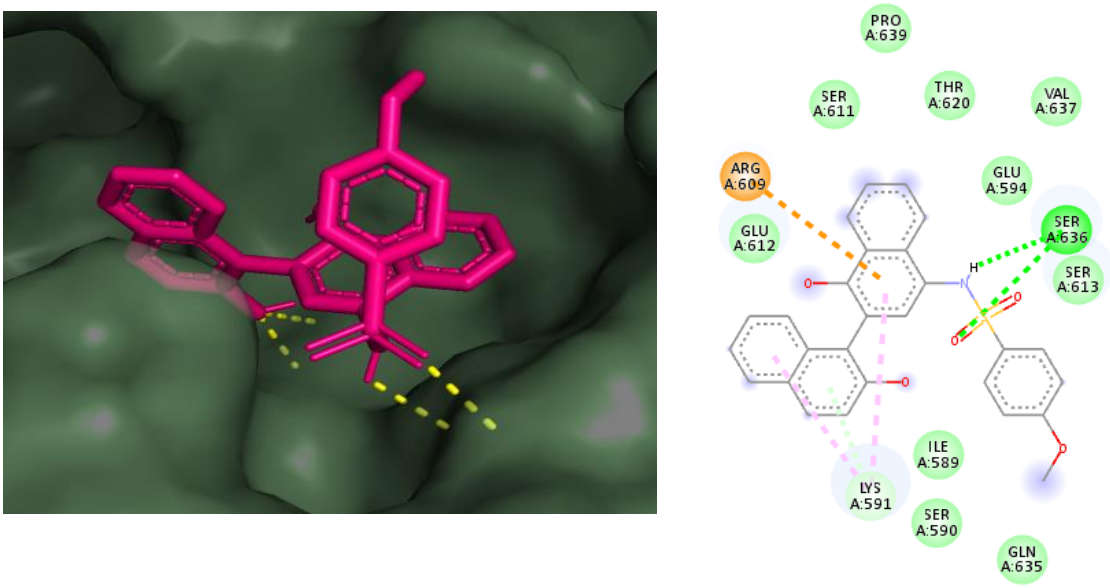


Figure 4.24. (a) TNF docked with its inhibitor shows a strong bonding due to the presence of many Van der Waal interactions, with overall -8.6 binding energy (b) TNF docked with plant compound 6, showing the lowest binding energy of -8.2. The presence of 3 hydrogen bonds provides stability to the complex.

(a)



(b)

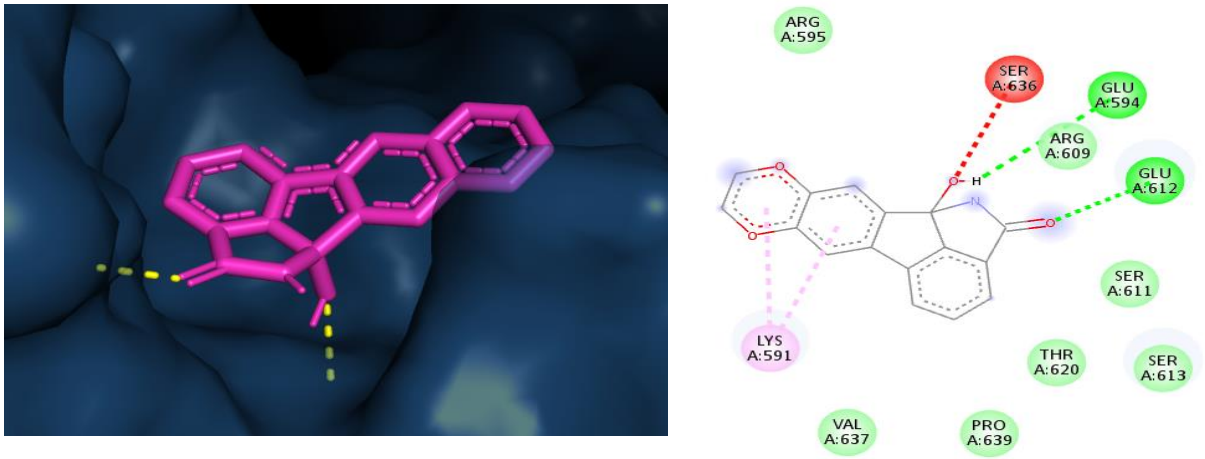
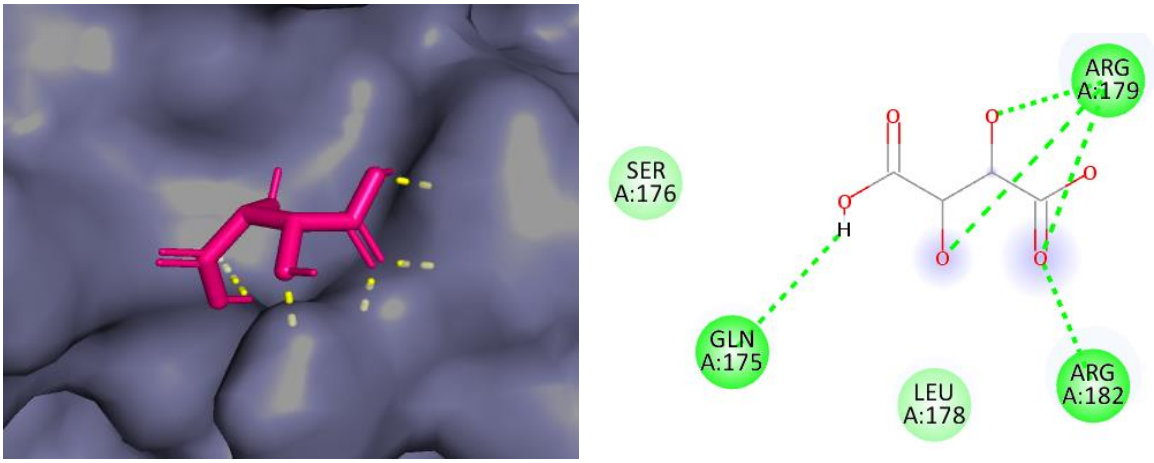


Figure 4.25. (a) STAT3 docked with inhibitor (TTI-101), with -7.2 binding energy (b) STAT3 docked with compound 7, having lowest binding energy i.e., -7.1.

(a)



(b)

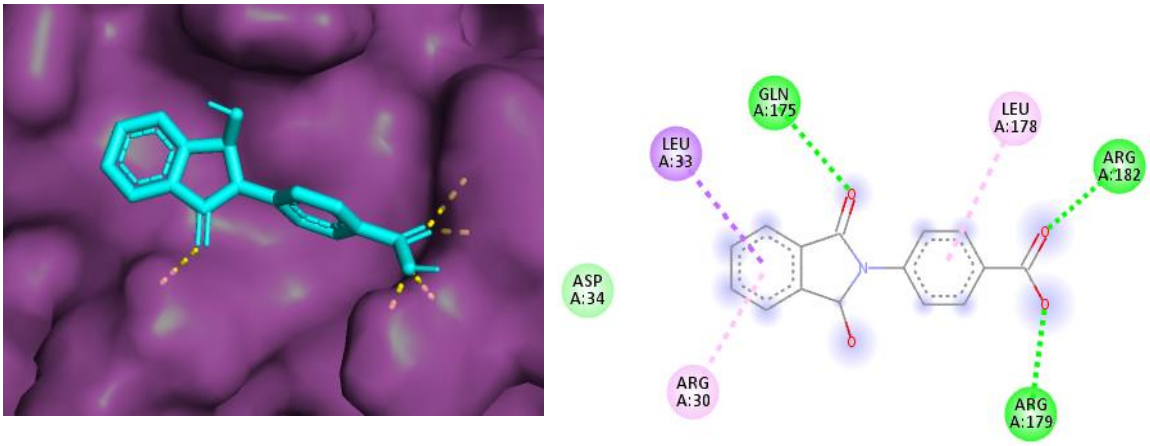


Figure 4.26. (a) IL-6 (1alu) docked with its inhibitor, with -3.7 binding energy (b) IL-6 (1alu) docked with compound 6, with lowest binding energy of -6.3.

4.13. MD Simulation Analysis

After successful molecular docking of all the three proteins, they were further analyzed through MD simulation.

I. The results showed that among all three proteins, TNF-ligand complex had the highest stability, and the ligand was highly intact throughout simulation. Therefore, it can be stated that compound number 6 named (4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl) benzoic acid) of *D. viscosa* is a good inhibitor against TNF-α.

II. According to the results of STAT3, selected plant compound 7 named (3-(2, 3-Dihydro-1, 4-benzodioxin-6-yl)-3-hydroxy-2H-isoindol-1-one) did not show any interactions with the protein after MD simulation, declaring that the compound was not a good fit for STAT3 protein.

III. Whereas, in the results of IL-6, little attachment between ligand 7 named (4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl) benzoic acid) and protein was observed.

4.13.1. TNF-α

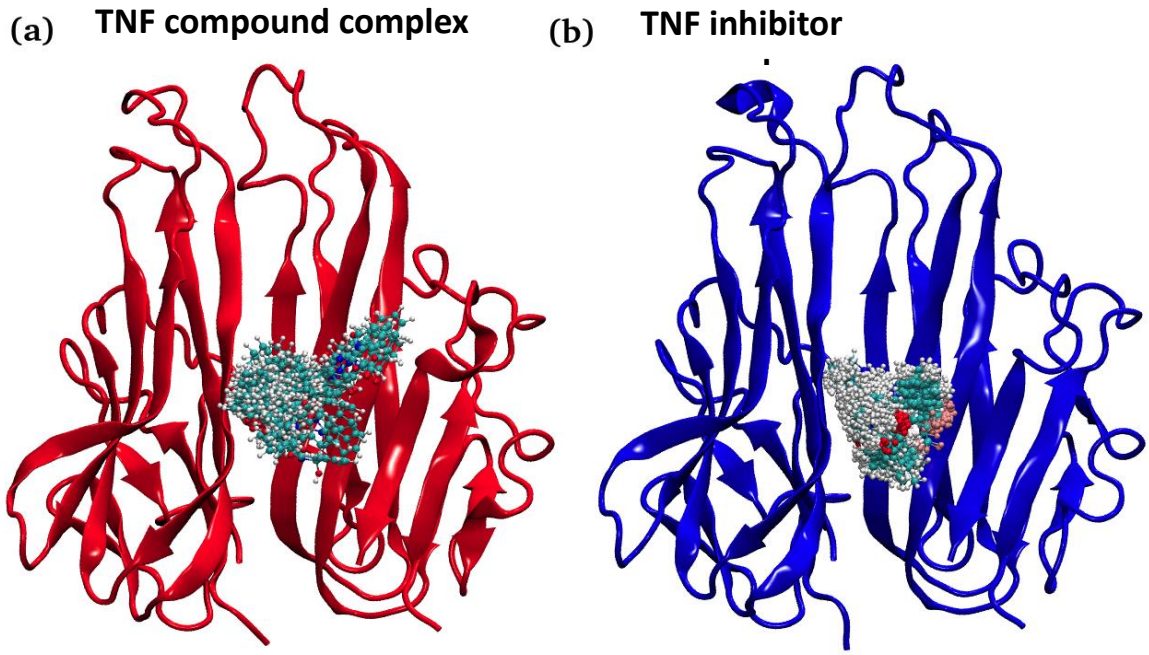


Figure 4.27. Simulation snapshots of ligand in (a) TNF compound complex and (b) TNF inhibitor complex systems. The ligand snapshots have been taken at every 1 ns of simulation time, while the protein is fixed at 0ns (initial structure). The ligand remained in a bound state in both systems, however, (a) is slightly flexible, occupying somewhat more space in the binding site, while (b) is highly compact.

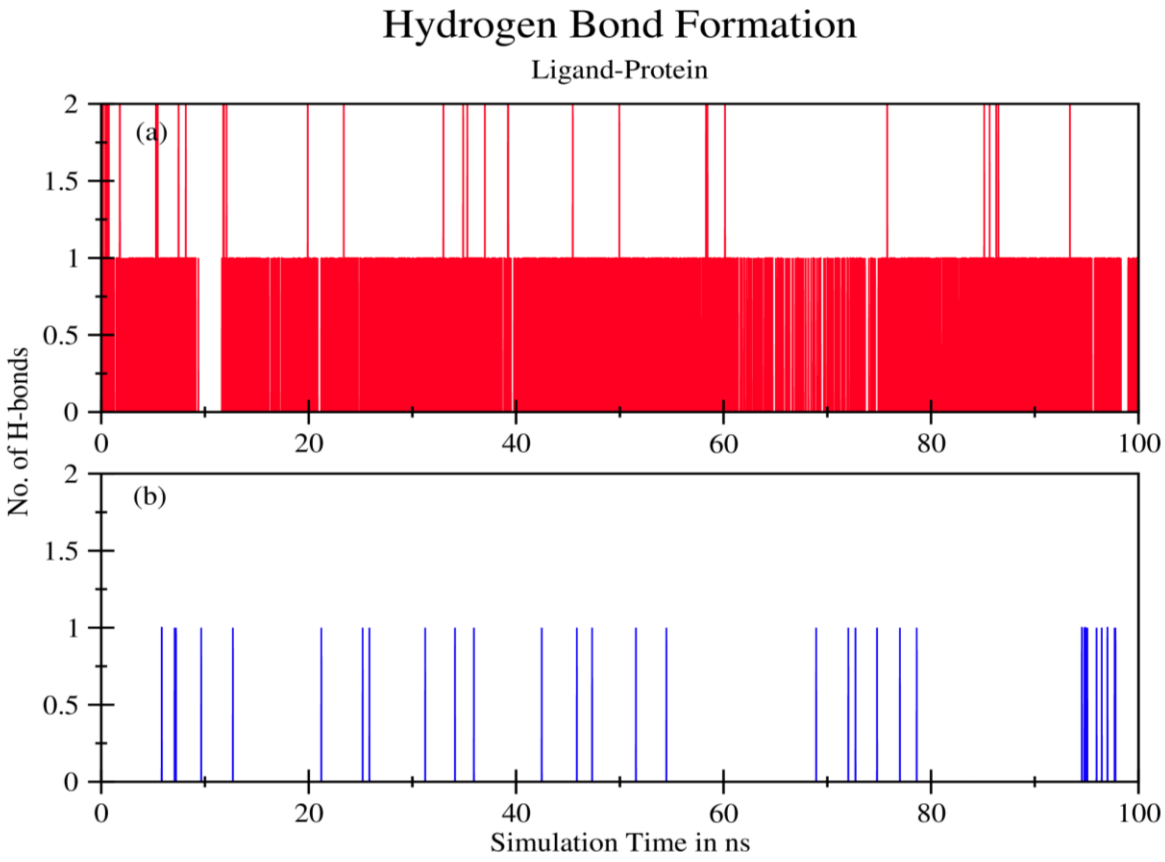


Figure 4.28. The total number of hydrogen bonds formed between ligand and protein during 100ns simulation time for (a) TNF compound complex and (b) TNF inhibitor complex systems. Ligand exhibits hydrogen bonds and forms consistent interactions with protein in (a) system. In (b) no significant H-bonds are observed.

RMSD (Protein)

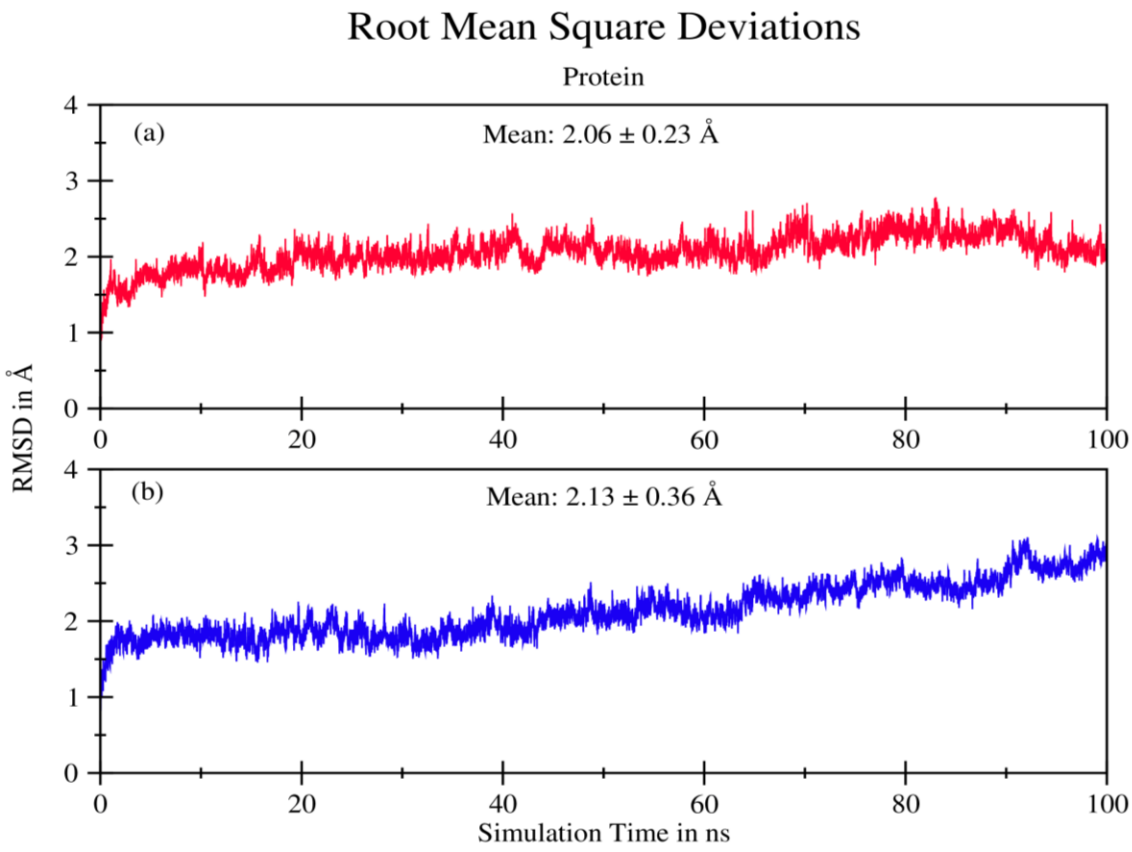


Figure 4.29. RMSD calculated for protein using 'C-alpha' atoms using the Bio3D module of the R program. (a) TNF_compound_complex and (b) TNF_inhibitor_complex systems. Overall, the RMSD is stable with various local conformational changes.

RMSD (Ligand)

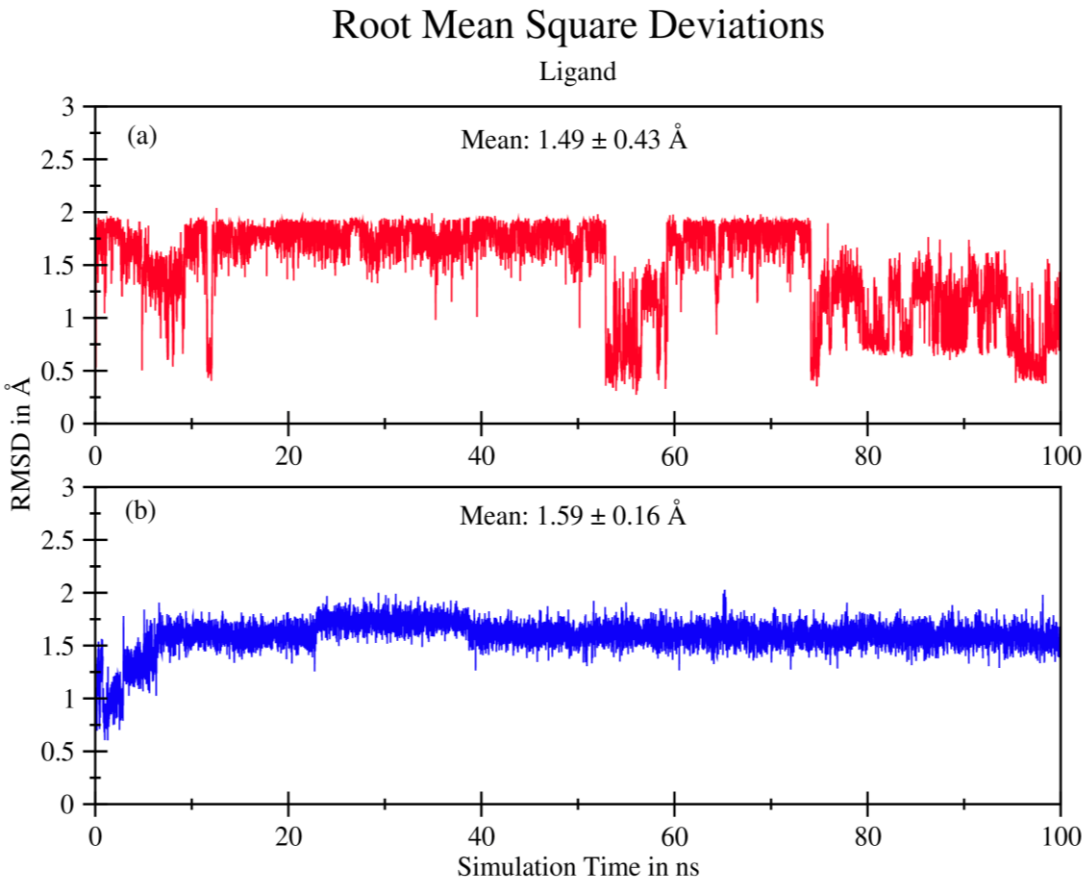


Figure 4.30. RMSD calculated for a ligand using the 'distance between ligand atoms' using the Bio3D module of the R program. (a) TNF_compound_complex and (b) TNF_inhibitor_complex systems. RMSD plot shows that the ligand exhibits conformational flexibility within the binding site, particularly at around 5-13ns, 43-60ns, and 74-100ns in (a). In (b) the ligand shows conformational flexibility at around 0-8 ns and remained very stable throughout the simulation.

RMSF

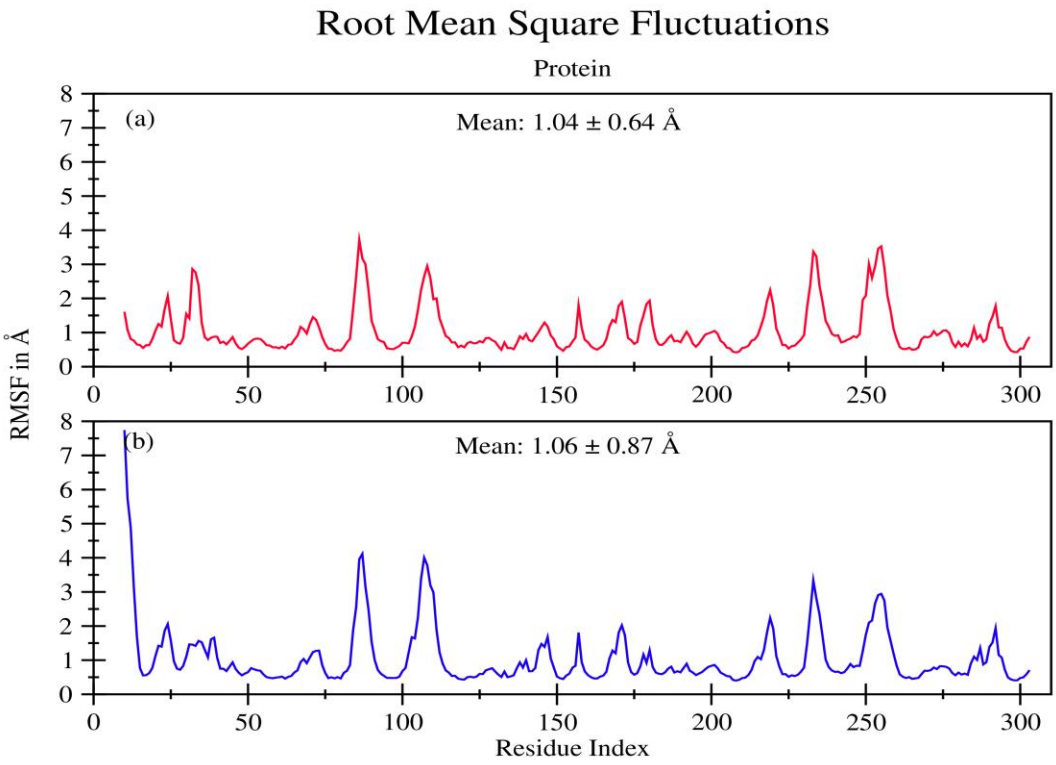


Figure 4.31. RMSF is calculated for proteins using 'C-alpha' atoms using the Bio3D module of the R program. (a) TNF_compound_complex and (b) TNF_inhibitor_complex systems. Overall, the system shows a significant fluctuation pattern in the plot. A higher fluctuation can be seen for residues 25 to 35, 80 to 90, 110 to 120, 230 to 240 and 250 to 265 in (a) while a similar pattern is observed along with C-terminal regions in (b).

Principal Component Analysis

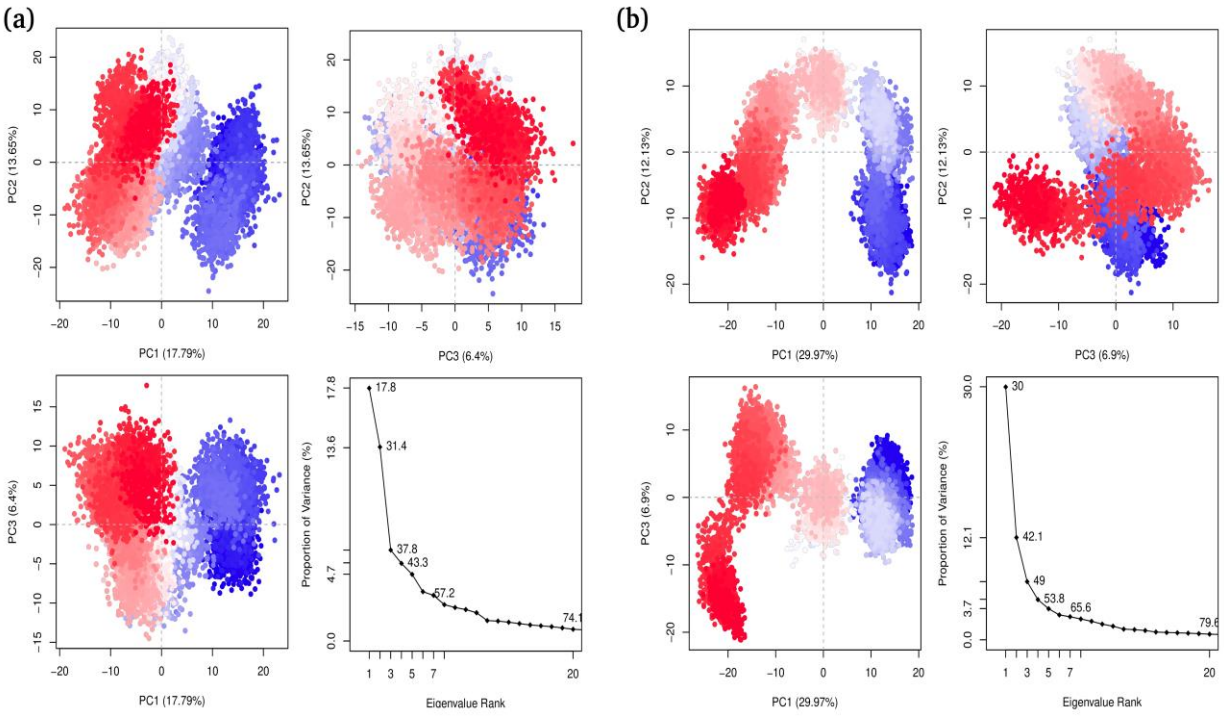


Figure 4.32. Principal Component Analysis of protein in (a) TNF_compound_complex and (b) TNF_inhibitor_complex systems were calculated from the Bio3D program of R. First three PCs capture 37.8% and 49.0% of the structural variance in complexes (a) and (b), respectively.

Table 4.7. Ligand-protein binding energy calculated from MMGBSA calculation. The major contribution is coming from van der Waal's interactions in both complexes; VDW is more favorable in TNF_inhibitor_complex; also indicated by weak H-bond numbers.

Individual Energy Contribution in Ligand-Protein binding from MMGBSA calculation	(a) TNF_compound_complex (Energy in kcal/mol)	(b) TNF_inhibitor_complex (Energy in kcal/mol)
$E^{van\ der\ Walls}$	-22.28	-47.28
$E^{Electrostatics}$	-8.04	-4.96
$E^{Generalized\ Born}$	19.08	28.03
E^{SURF}	-2.89	-5.62
ΔG	-14.14	-29.82

4.13.2. STAT3

Ligand-bound conformations

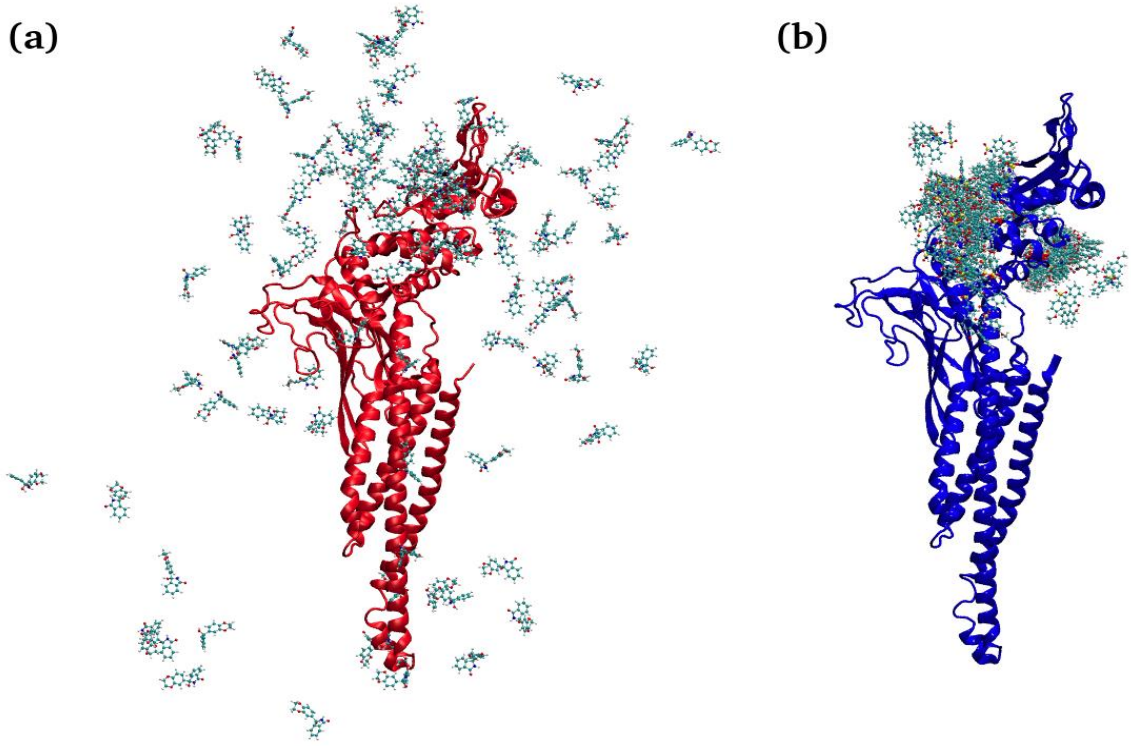


Figure 4.33. Simulation snapshots of ligand in (a) STAT3_compound_complex and (b) STAT3_inhibitor_complex systems. The ligand snapshots have been taken at every 1 ns of simulation time, while the protein is fixed at 0ns (initial structure). The ligand leaves the binding site in both systems, however, ligand in (b) is still present nearby the binding site.

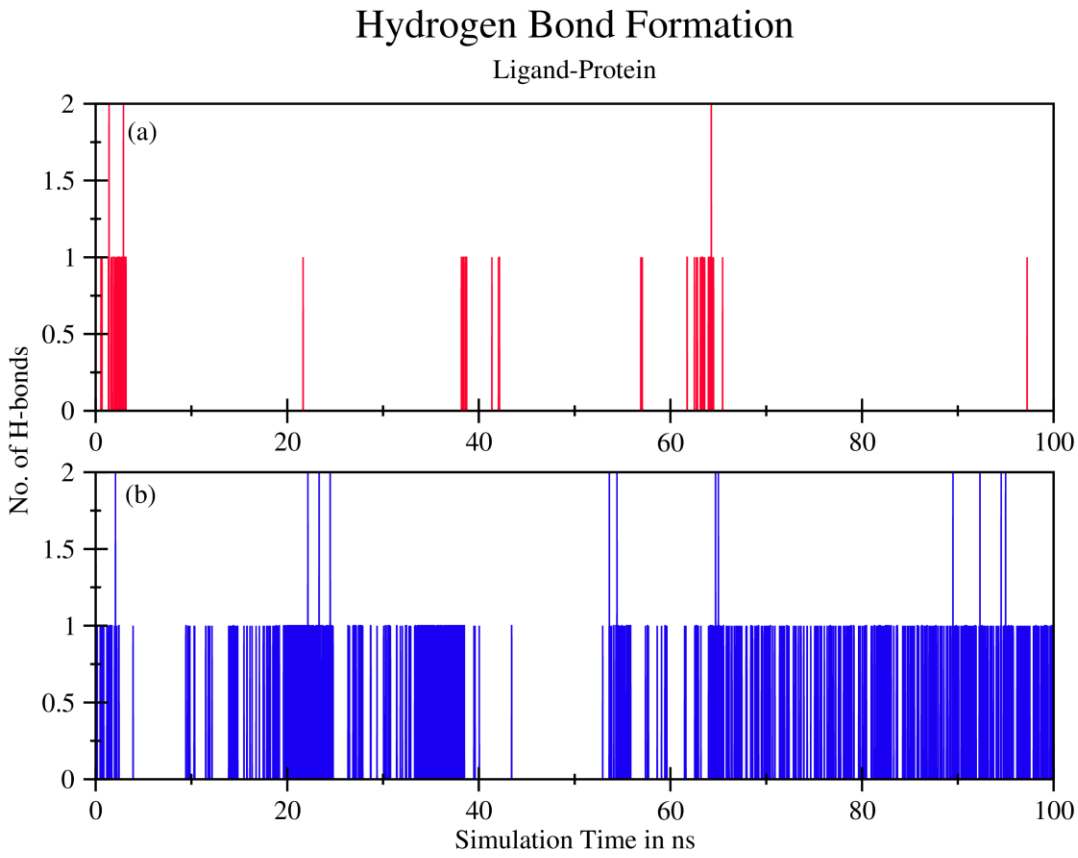


Figure 4.34. Total number of hydrogen bonds formed between ligand and protein during 100ns simulation time for (a) STAT3_compound_complex and (b) STAT3_inhibitor_complex systems. Ligand does not exhibit hydrogen bonds with protein in (a) while shows some consistent interactions with protein in (b). However, in (b) ligand does not form any H-bonds during 3-10ns, and 40-53ns indicating that ligand might have left the site.

RMSD (Protein)

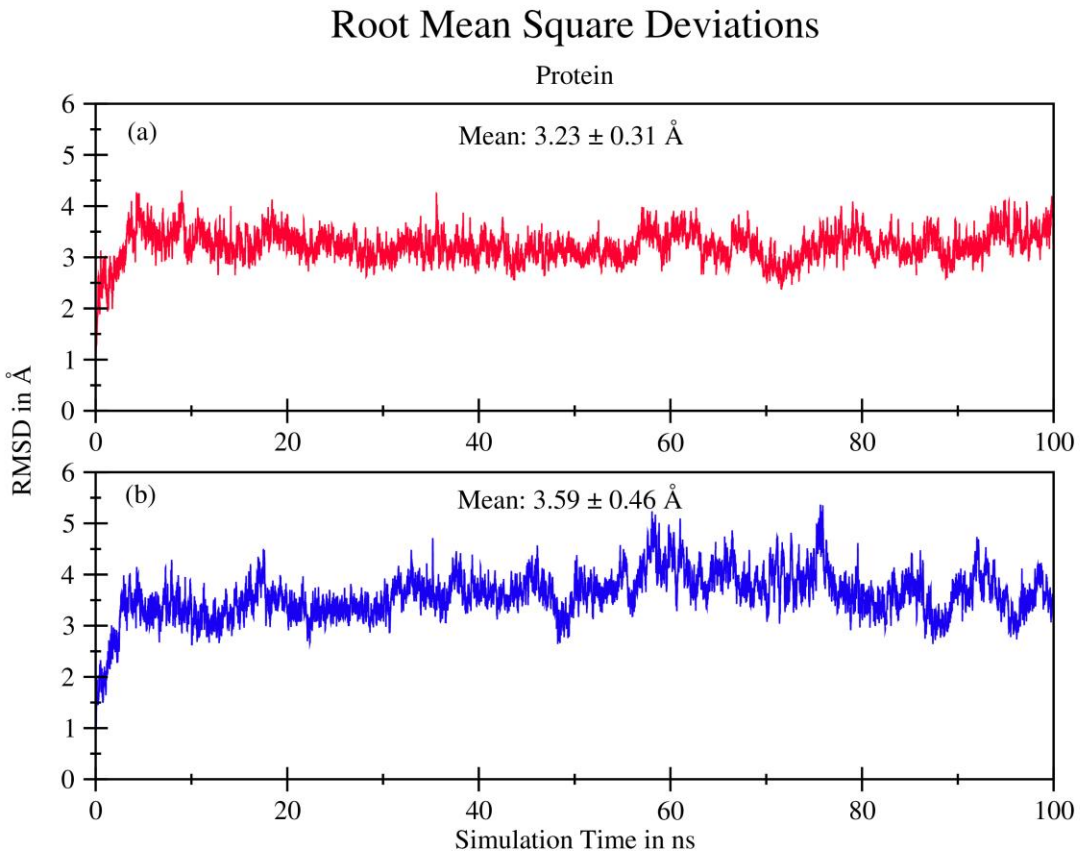


Figure 4.35. RMSD calculated for protein using 'C-alpha' atoms using Bio3D module of R program. (a) STAT3_compound_complex and (b) STAT3_inhibitor_complex systems. Overall, the RMSD is very stable with various small local conformational changes.

RMSD (Ligand)

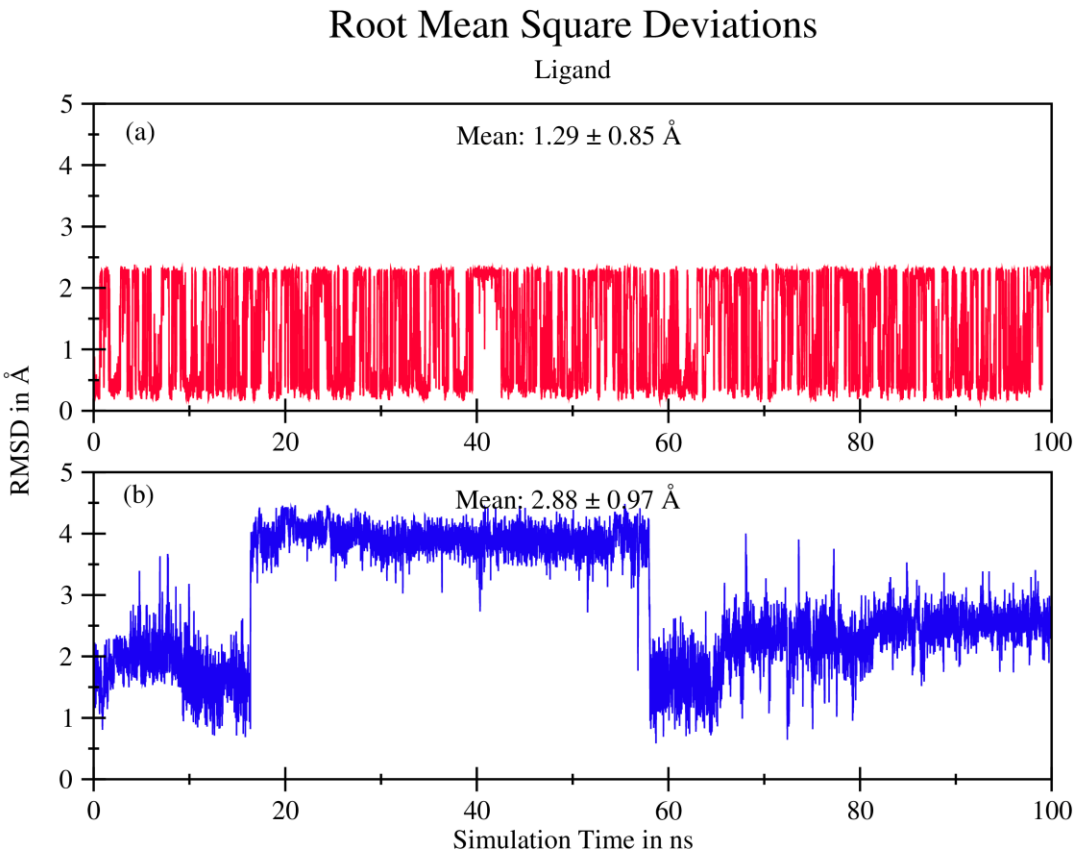


Figure 4.36. RMSD calculated for ligand using 'distance between ligand atoms' using Bio3D module of R program. (a) STAT3_compound_complex and (b) STAT3_inhibitor_complex systems. RMSD plot shows the ligand exhibits incredibly significant conformational flexibility in (a) and within the binding site particularly at around 18-58ns in (b).

RMSF

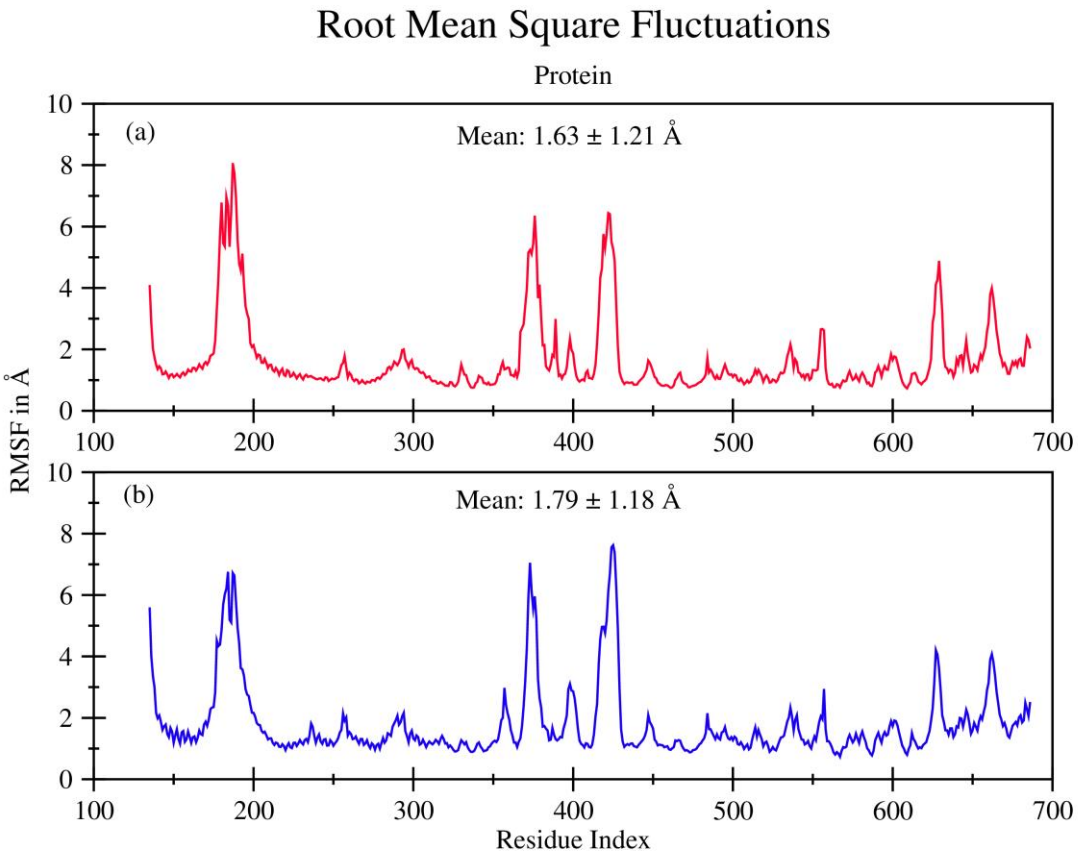


Figure 4.37. RMSF calculated for proteins using 'C-alpha' atoms using Bio3D module of R program. (a) STAT3_compound_complex and (b) STAT3_inhibitor_complex systems. Overall, the system shows significant fluctuation pattern in the plot. A higher fluctuation can be seen for residues 170 to 200, 370 to 380, and 420 to 430 in both (a) and in (b). The N terminal is comparatively more flexible than the C terminal.

Principal Component Analysis

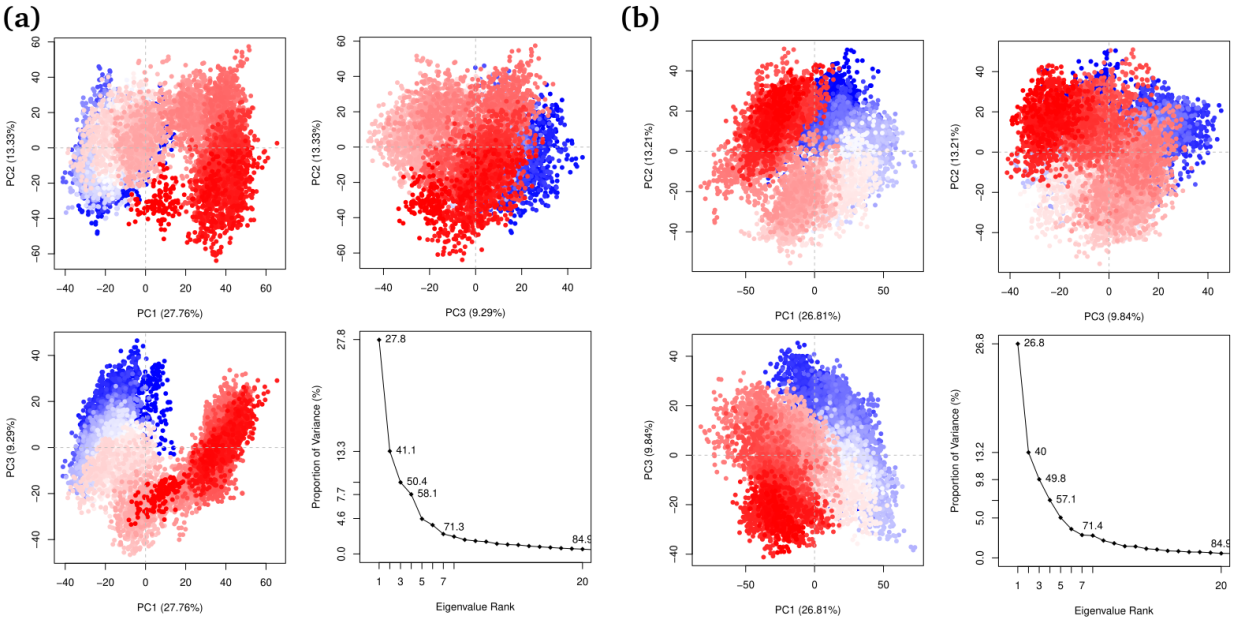


Figure 4.38. Principal Component analysis of protein in (a) STAT3_compound_complex and (b) STAT3_inhibitor_complex systems was calculated from Bio3D program of R. First three PCs capture 50.4% and 49.8% of structural variance in complexes (a) and (b), respectively.

Table 4.8. Ligand-protein binding energy calculated from MMGBSA calculation. The major contribution is coming from van der Waal's interactions in STAT3_Inhibitor complex. STAT3_Compound does not bind with protein.

Individual Energy Contribution in Ligand-Protein binding from MMGBSA calculation	(a) STAT3_Compound _complex (Energy in kcal/mol)	(b) STAT3_Inhibitor _complex (Energy in kcal/mol)
$E^{van\ der\ Walls}$	-1.58	-18.27
$E^{\ Electrostatics}$	-1.22	-8.73
$E^{\ Generalized\ Born}$	2.59	16.72
E^{SURF}	-0.23	-2.37
ΔG	-0.44	-12.65

4.13.3. IL-6

Ligand-bound conformations

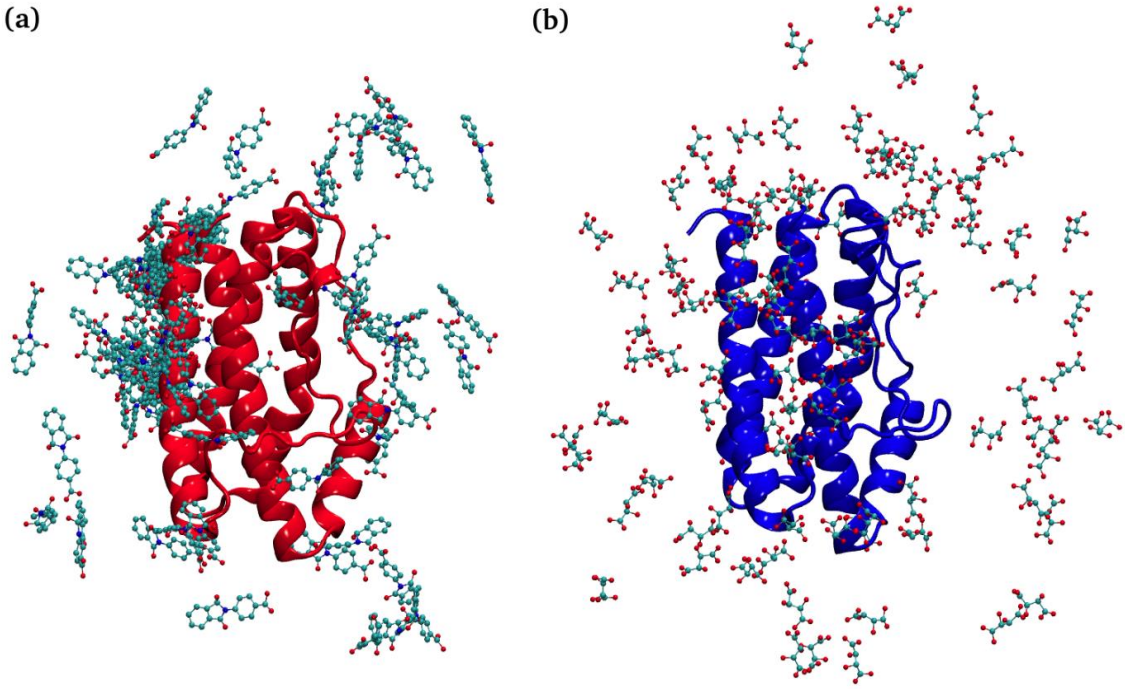


Figure 4.39. Simulation snapshots of ligand in (a) IL-6_plant_complex and (b) IL-6_inhibitor_complex systems. The ligand snapshots have been taken at every 1 ns of simulation time, while the protein is fixed at 0ns (initial structure). It can be observed that the ligand does not bind with the protein in both systems.

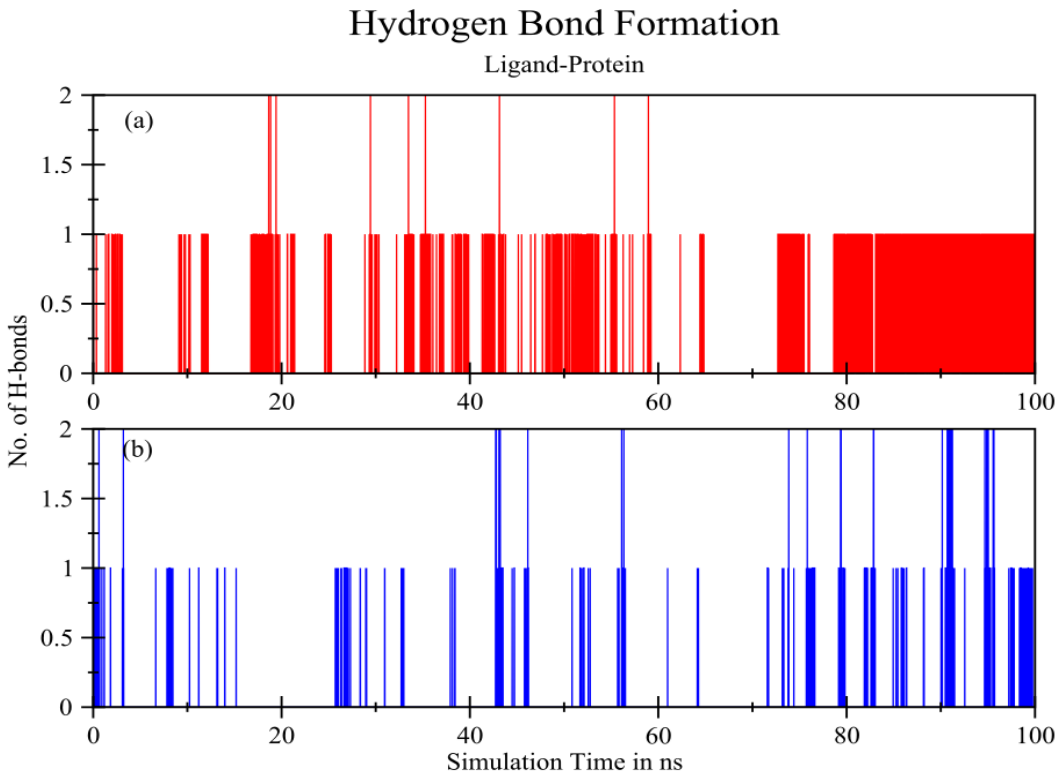


Figure 4.40. Total number of hydrogen bonds formed between ligand and protein during 100ns simulation time for (a) IL-6_plant_complex and (b) IL-6_inhibitor_complex systems. Ligand exhibits non-consistent and smaller number of hydrogen bonds with protein in (a) system until 80ns, indicating that ligand may not be binding with protein. After 80ns the H-bond become consistent. In (b) no significant H-bonds are observed indicating no ligand binding with protein.

RMSD (Protein)

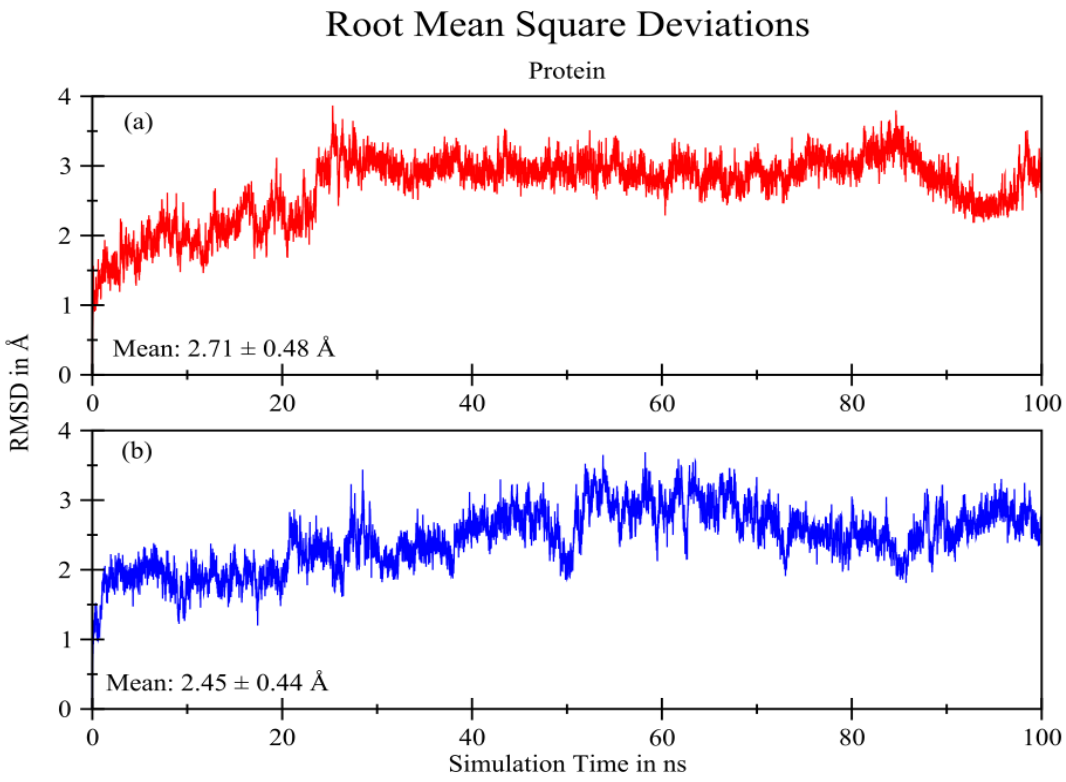


Figure 4.41. RMSD calculated for protein using 'C-alpha' atoms using Bio3D module of R program. (a) IL-6_plant_complex and (b) IL-6_inhibitor_complex systems. Overall, the RMSD is stable with various local conformational changes.

RMSD (Ligand)

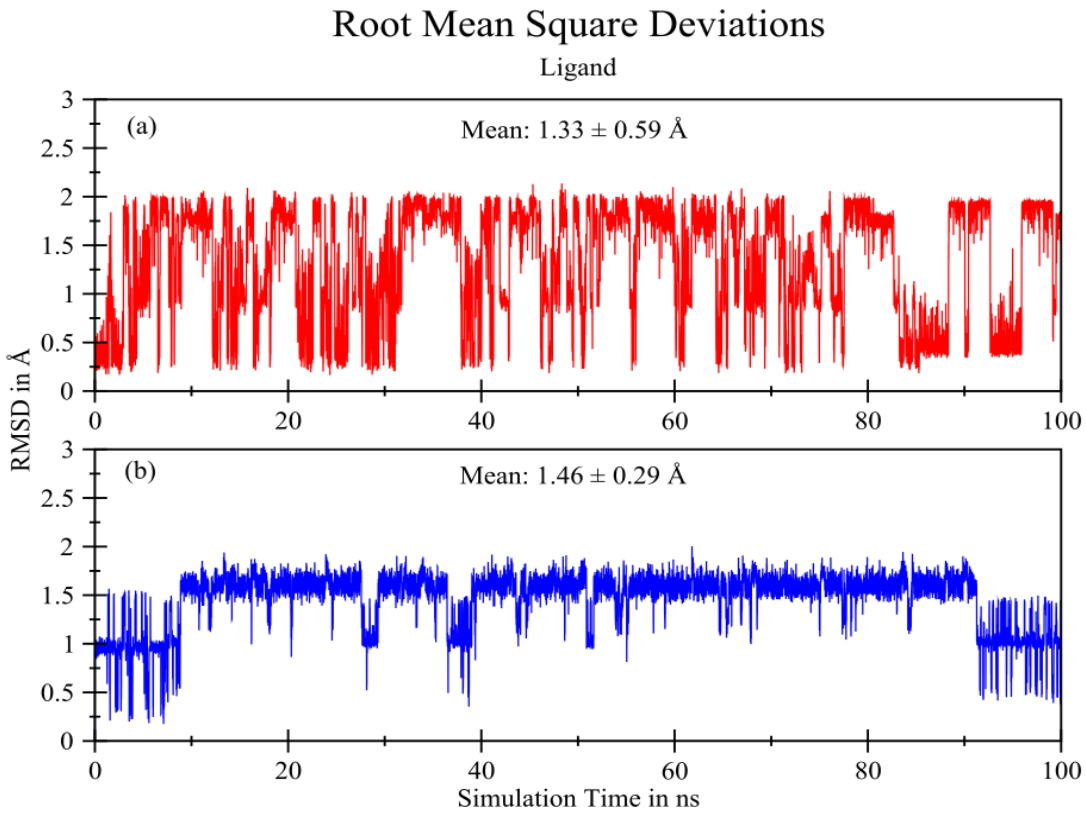


Figure 4.42. RMSD calculated for ligand using 'distance between ligand atoms' using Bio3D module of R program. (a) IL-6_plant_complex and (b) IL-6_inhibitor_complex systems. RMSD plot shows the ligand exhibits great conformational flexibility throughout the simulation in (a) and comparatively smaller flexibility particularly at around 10ns, 22ns, 37ns and 90ns, in (b), as also indicated by its lower standard deviation value.

RMSF

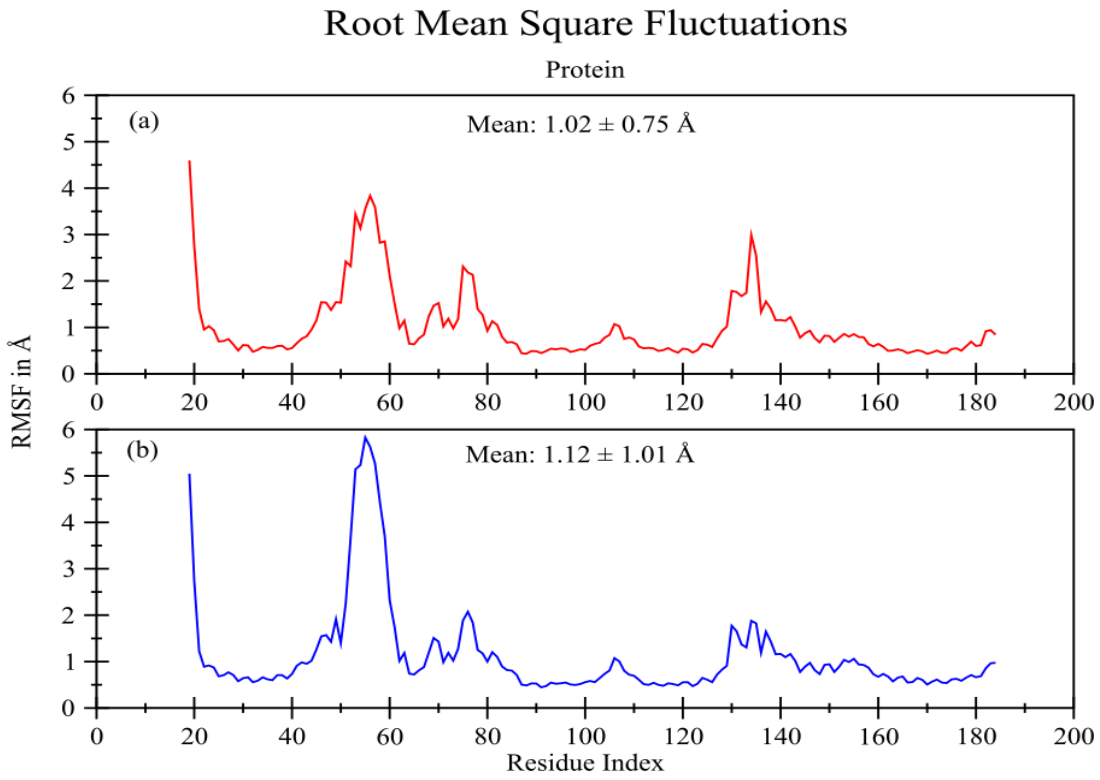


Figure 4.43. RMSF calculated for proteins using 'C-alpha' atoms using Bio3D module of R program. (a) IL-6_plant_complex and (b) IL-6_inhibitor_complex systems. Overall, the system shows significant fluctuation pattern in the plot. A higher fluctuation can be seen for residues N-terminal, 41 to 65, 66 to 80, and 127 to 139, in both (a) and (b). C-terminal remained very rigid in both complexes.

Principal Component Analysis

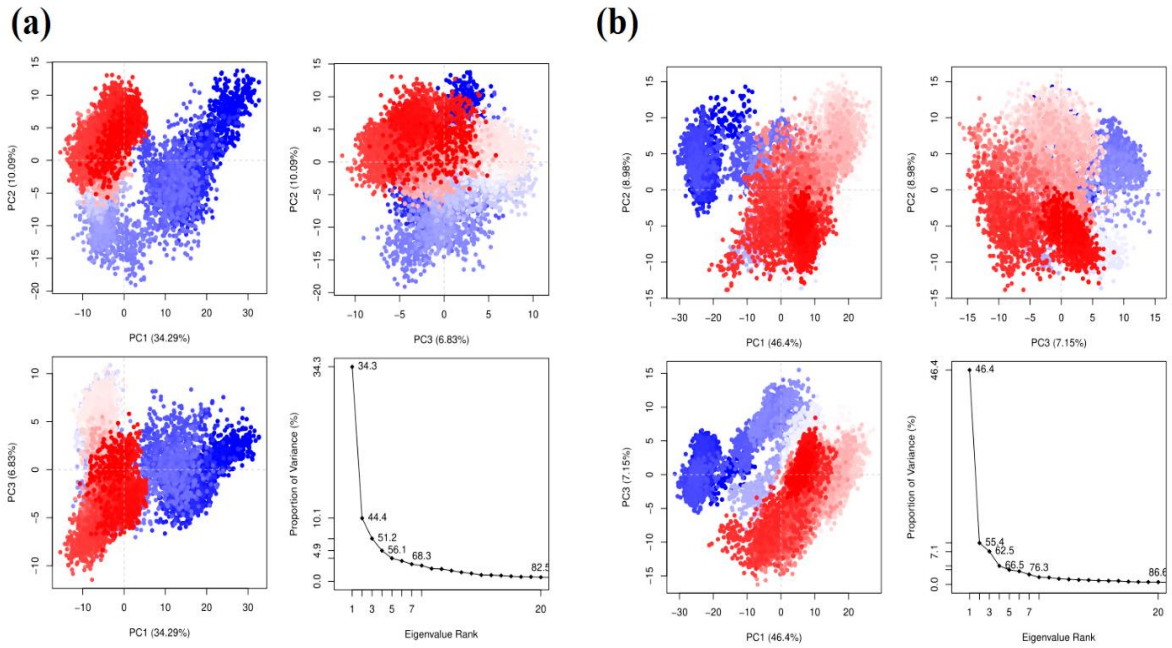


Figure 4.44. Principal Component analysis of protein in (a) IL-6_plant_complex and (b) IL-6_inhibitor_complex systems was calculated from Bio3D program of R. First three PCs capture 51.2% and 62.5% of structural variance in complexes (a) and (b), respectively.

Table 4.9. Ligand-protein binding energy calculated from MMGBSA calculation. The major contribution is coming from van der Waal's interactions and electrostatics in (a) IL-6_Plant_complex; also indicated by weak H-bond numbers. In (b) IL-6_Inhibitor _complex overall binding energy is not favorable.

Individual Energy Contribution in Ligand-Protein binding from MMGBSA calculation	(a) IL-6_plant _complex (Energy in kcal/mol)	(b) IL-6_Inhibitor _complex (Energy in kcal/mol)
$E^{van\ der\ Walls}$	-9.32	-1.16
$E^{Electrostatics}$	-7.28	-2.47
$E^{Generalized\ Born}$	12.87	4.15
E^{SURF}	-1.46	-0.26
ΔG	-5.19	0.25

5. DISCUSSION

Rheumatoid arthritis is a chronic inflammatory disease that destroys cartilage and joints. It causes swelling of the synovial lining, leading to immobility. The precise etiology of RA is still unclear, but it is well-known to be caused by genetic and environmental factors. The disease is observed to be three times more common in females as compared to males. According to the WHO, it affects 0.3-1 percent of the global population. Whereas, in Pakistan, the prevalence ranges from 0.5 to 1.9 percent. Many treatment options are availed for its treatment including NSAIDs, DMARDs, and biologics. These treatments help in alleviating disease symptoms such as pain and swelling. However, their adverse side effects have limited their use. Therefore, researchers are exploring herbal plants as a new alternative that minimizes the side effects, are non-toxic and cost-effective with high therapeutic potential against RA.

The therapeutic potential of a plant is based on the presence of its bioactive compounds. These are also known as "Phytochemicals." These provide various vital natural properties to plants because of which they show incredible pharmacological effectiveness. According to many studies, phytochemicals are said to have excellent anti-inflammatory properties with few side effects (Nandakumar et al., 2018).

The main aim of the current study was to identify and evaluate bioactive compounds of *Dodonaea viscosa* for their therapeutic potential against rheumatoid arthritis. The *Dodonaea* genus has a variety of traditional and modern applications. They are high in phytochemical constituents. *D. viscosa* belongs to the Sapindaceae family. It is an ethnobotanical plant that possesses exceptional medicinal and therapeutic properties, with both traditional and modern applications (*(PDF) Dodonaea viscosa Linn Used Disease by Irula Tribes Kanchipuram District Tamil Nadu, India*, n.d.; Beshah et al., 2020).

It has been used in Traditional Chinese Medicine (TCM) for treating multiple health conditions and for various other purposes. It has been used as a folk remedy for a variety of health problems, including rheumatoid arthritis and joint pain. Various parts of the plant are used as antiviral, analgesic, anti-inflammatory, anti-microbial, and laxatives due to the presence of various therapeutic properties (*Historical Origin, Chemical*

Constituents and Therapeutic Potentials of Sanatha (Dodonaea Viscose) - a Brief Review., n.d.).

The plant is reported to have anti-inflammatory, antioxidant, anti-microbial, cytotoxic, wound healing, and antispasmodic potentials. (*[PDF] Antimicrobial, Antioxidant and Anticancerous Studies on Dodonaea viscosa Leaf Extracts Against Human Breast Cancer Cell Line (MCF-7) | Semantic Scholar*, n.d.; Al-Snafi, 2017; Herrera-Calderon et al., 2020b; Nayeem et al., 2021)

Several studies have reported the bioactive compounds of *D. viscosa*, but many more compounds are yet to be explored by scientists. Among the reported compounds, Hautriwaic acid and viscosine are the most common ones. Hautriwaic acid acts as a hepatoprotective agent whereas, viscosine acts as a lipoxygenase inhibitor. (Ali et al., 2014; A. Z. Khan et al., 2013)

To obtain our objective, several phytochemical tests were performed on an ethanol extract of *D. viscosa*. The results were positive for alkaloids, phenols, flavonoids, tannins, coumarins, terpenoids, sterols, saponins, steroids, and cardiac glycosides. Whereas, negative results were shown for glycosides, anthraquinones, and anthocyanins. Similar results of the phytochemical analysis were observed in a few other studies. (Priyankadevi & Arunprasath, 2018; Saranya & Divyabharathi, n.d.; Venkatesh et al., 2008)

The antioxidant potential of *D. viscosa* leaves (ethanol extract) was evaluated by two methods including **(1)** 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and **(2)** Ferric Reducing Antioxidant Power (FRAP) assay. The results for DPPH indicated that *D. viscosa* possesses a significantly higher antioxidant potential as compared to ascorbic acid, which is the standard. The results were significant with a P-value of 0.0018, which is less than 0.05. Two studies from 2018 and 2011 also reported similar results for DPPH activity of *D. viscosa* (Priyankadevi & Arunprasath, 2018; Riaz et al., 2012)

The results of the FRAP assay indicated that the ferric reducing potential of the plant increased with the increasing concentration. The results were highly significant with a P-value of 1.20E-05. The trend line in the graphs demonstrates the extract's antioxidant

activity, which increased with concentration. Therefore, both assays demonstrate that *D. viscosa* has a good antioxidant potential.

The anti-inflammatory potential was evaluated for ethanol extract of *D. viscosa* leaves by two *in vitro* methods including **(1)** Protein denaturation and **(2)** Human Red Blood Cell (HRBC) Membrane stabilization assay. The results of protein denaturation indicated that plant extract had higher percentage inhibition at 600 nm as compared to standard, diclofenac potassium. After statistical analysis, linear regression gave a significant P-value of 0.01897. This demonstrates that the ethanol extract was effective in inhibiting the denaturation of protein. As it is a common aspect of RA progression. Therefore, proving the anti-inflammatory potential of *D. viscosa* leaves. Similar results were seen in a study for evaluating in vitro biological activity of *D. viscosa* leaves (Nayeem et al., 2019).

The results of the HRBC membrane stabilization assay demonstrated that plant extract had a higher percentage inhibition as compared to standard at 620 nm. The results were significant with a P-value of 0.00132, which is lower than 0.05. Therefore, extract of *D. viscosa* plays a vital role in the stabilization of cellular membranes which get affected during the disease prognosis. After that, total phenolic content (TPC) and total flavonoid content (TFC) both were calculated for the ethanol extract to obtain the quantitative phytochemical analysis which indicated the antioxidant potential of *D. viscosa*.

Drug development has undergone a remarkable transformation due to in silico analysis and bioinformatics techniques. It has cut down the time and money needed to develop new medications. The second stage of the current investigation involved determining which compounds of *D. viscosa* have therapeutic potential against the development of RA. Therefore, GC-MS analysis generated a library of 480 compounds. For the evaluation of drug-like qualities, five separate rules—the Lipinski rule of five, the Ghose rule, the Muegge rule, the Egan rule, and the Veber rule—were considered. Each of these rules assesses drug-like qualities based on various criteria. Among these 480 compounds, only 144 followed these five rules. The compounds were then examined further for molecular weight and ADMET characteristics, i.e., HERG, oral bioavailability, TPSA, BBB, cytotoxicity, and carcinogenicity.

This shortlisting gave the following nine final compounds **(1)** 7-(2-Fluorophenyl)-4H,7H-[1,2,4]triazolo[1,5-a]pyrimidine-5-carboxylic acid **(2)** 4-Piperidinecarboxamide, 1-[2-(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]- **(3)** 2-(4-Hydroxypyrimidin-2-ylsulfanyl)-N-(5-methylisoxazol-3-yl)acetamide **(4)** Acetamide, N-methyl-2-[(5-methyl-1,3,4-thiadiazol-2-yl)thio]-N-phenyl **(5)** 5-(p-Acetylaminophenylsulfonyl)dihydro-1,3,5-dioxazine (6) 4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl)benzoic acid **(7)** 3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-hydroxy-2H-isoindol-1-one **(8)** 4-Acetyl-5-(furan-2-yl)-3-hydroxy-1-(pyridin-3-yl)-5H-pyrrol-2-one **(9)** 5,6,7-Trimethyl-[1,2,4]triazolo[1,5-a]pyrimidin-2-ylsulfanyl)-acetic acid.

Therapeutic targets of RA were retrieved from the CTD database. After that PPI network was developed on STRING software which was then exported to cystoscope extension "Cytohubba" for HUB gene analysis. Various parameters were applied to obtain the top ten genes or nodes. From these top ten genes, the top three were selected based on their node scores. This means that the genes/nodes that fulfill the maximum parameters of Cytohubba were selected. These shortlisted targets included TNF-α with a score of ten out of eleven parameters. Similarly, STAT3 and IL-6 with a score of eight out of eleven parameters. These genes were studied from literature for their pathways involved in RA prognosis from KEGG pathways, their binding domains (active sites), their cellular location, and their mode of action.

All these targets were docked with shortlisted phytochemicals and the best-docked complexes were selected for further analysis. The best protein-ligand complexes had the lowest binding energies which indicate the stability of the complex. Among all three selected genes, TNF-α showed the best results with the lowest binding energy of -8.2 (plant compounds). Multiple studies have reported similar results that have novel small molecule inhibitors of TNF-α that directly bind and inhibit the progression of RA and other autoimmune diseases from various natural sources (Bai et al., 2021; Zaka et al., 2018).

The best-docked complexes were further analyzed by MD simulations. This provides a real-time three-dimensional environment to the protein with various parameters like specific temperature, pressure, presence of water molecules and ions, etc. The results

showed that TNF-α was perfectly docked with plant compound, and it remained intact throughout the simulation process with slight flexibility within the ligand binding site. Therefore, this compound can act as a competitive therapeutic agent. Whereas no interaction was seen after 100ns simulation for STAT3, and little interaction was observed for IL-6, thus we can use derivatives of IL-6 in the future.

6. CONCLUSION

The study proved that *Dodonaea viscosa* had good anti-rheumatic potential. The in vitro studies concluded that the phytochemicals of the plant had remarkable antioxidant and anti-inflammatory potential that helped in hampering RA prognosis. The phytochemicals of *D. viscosa* showed a high potential to be developed into an anti-rheumatic medication after further testing. Therefore, *in silico* studies were performed to evaluate the anti-rheumatic potential of all compounds of *D. viscosa* and it is concluded that *D. viscosa* have certain bioactive compounds with the potential to target arthritis-related proteins. In the current study, the plant compound named 4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl) benzoic acid (PubChem 18873897), showed excellent inhibitory potential against TNF-α, which plays a significant role in the disease. However, additional in-vitro and in-vivo testing is strongly advised to assess the efficacy of nine compounds in alleviating the effects of Rheumatoid arthritis progression.

FUTURE PROSPECTS

Compounds from *Dodonaea viscosa* have the potential to target rheumatoid arthritis-related proteins. They can be evaluated for their role in other proteins as well. In-vitro and in-vivo investigations are strongly advised to assess the effectiveness of compound 6 named 4-(1-Hydroxy-3-oxo-1H-isoindol-2-yl) benzoic acid which proved to be a good inhibitor against TNF-α, playing a role in reducing the impacts of rheumatoid arthritis progression. Derivatives of potential compounds can also be used to check their binding affinity and observe their mode of action. Furthermore, candidate therapeutic drugs can be evaluated in cells taken from RA patients.

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