5.1 Introduction to *In-silico* Studies

In past few decades, the area of drug discovery has witnessed a rapid shift from conventional drug designing to the computer-based In-silico methods of drug discovery. In-silico studies, aims to predict and analyse the interactions between drugs and biological targets using computational technique to identify and optimize the hit to lead process of drug discovery. In-silico approaches have proven to be of greater help in screening large compound libraries at once, can be applied to drug-target and toxicity predictions and most importantly, relieve the pressure concerning the cost of laboratory works and animal sacrifices. [1] These computational screening has been proven to be essential at different phases of drug development process, fulfilling certain needs and providing with several advantages like efficient lead identification, cost effectiveness, target specificity and selectivity, exploring vast compound library, SAR and QSAR analysis, drug target identification, hit to lead optimization etc.

Computer Aided Drug Designs (CADD) has proven to reduce the number of chemical compounds that were to be experimentally monitored and evaluated earlier whereas it has increased the probability of success of a drug entity by discarding the inefficient, toxic, and pharmacologically inactive drug molecule. CADD approaches includes both Structure based drug designing and Ligand based drug designing. [2]

The *In-silico* drug design is the basically based on two main approaches first being the Structure Based Drug designing (SBDD) and the other being Ligand Based Drug Designing (LBDD) the former one deals with determining the structural information of the protein target experimentally by using nuclear magnetic resonance or X-ray crystallography, after knowing the structure of the target protein the structure-based virtual screening is carried out by molecular docking, For the second approach it requires a pre information of the inbuilt active ligand of the target protein about their structure, the active heterocyclic ring, the types of bonding, the electron doner and acceptor present in the structure. A common data base is then created collecting other drug compounds like the inbuilt ligand unless the target is novel. [2]

These *In-silico* approaches include various computational methods like two-dimensional qualitative structure activity relationship (2D QSAR), three-dimensional structure activity relationship (3D QSAR), pharmacophore modelling, virtual screening, molecular docking, molecular dynamic simulations (MDS) etc.

Conventional drug discovery and development are risky, time-consuming processes that include target identification and validation, lead compound discovery and optimization, and preclinical and clinical trials. Typically, *in vivo* and *in vitro* techniques are employed to examine drug safety, including adverse effects and toxicity. Recent advancements in *in vitro* models, such as organ-on-chip technology, have accelerated ADME-Tox assessments. However, these approaches remain time-consuming, labor-intensive, and costly. High-throughput screening (HTS) methods have been developed to accelerate the identification of pharmacologically active chemical compounds from large numbers of molecules using automated assays. Although automatic HTS systems reduce the need for human intervention, the scale of HTS remains low compared to the diversity of chemical structures. In addition, automated instruments remain expensive.

Recently, computer-aided drug discovery (CADD) approaches are attracting increasing attention as they can help mitigate the scale, time, and cost issues faced by conventional experimental approaches. CADD includes computational identification of potential drug targets, virtual screening of large chemical libraries for effective drug candidates, further optimization of candidate compounds, and *in silico* assessment of their potential toxicity. After these processes are conducted computationally, candidate compounds are subjected to *in* vitro/in vivo experiments for confirmation. Thus, CADD approaches can reduce the number of chemical compounds that must be evaluated experimentally while increasing the success rate by removing inefficient and toxic chemical compounds from consideration. To date, CADD has been successfully employed to bring new drug compounds to market for diverse diseases, including human immunodeficiency virus (HIV)-1-inhibiting drugs (atazanavir, saquinavir, indinavir, and ritonavir), anti-cancer drugs (raltitrexed), and antibiotics (norfloxacin).

Computer-aided drug design (CADD) methodologies are playing an ever-increasing role in drug discovery that are critical in the cost-effective identification of promising drug candidates. These computational methods are relevant in limiting the use of animal models in pharmacological research, for aiding the rational design of novel and safe drug candidates, and for repositioning marketed drugs, supporting medicinal chemists and pharmacologists during the drug discovery trajectory.

5.1.1 Molecular docking

Molecular docking is an algorithm based widely used computational technique in drug discovery that simulates and predicts the conformational space of the ligands or the newly designed drug molecule within the binding pocket of targeted protein by calculating their electrostatic interactions, van der Waals forces, hydrogen bonding and desolvation energy. Docking technique can generally be of two types namely rigid docking and flexible docking, In rigid docking the internal geometry of both receptor and ligand are considered to be a impliable with no rotational or conformational changes whereas in flexible docking the bonds of molecules (generally of smaller molecule i.e. ligand) are flexible that is they are free to rotate, here the docking is carried out and energy is calculated for each pose out of which the most suitable or optimum pose is selected. Along with protein-ligand, protein-nucleotide docking method can also assist in predicting protein-protein interactions and evaluating the affinity of complexes, thus enabling a better understanding of signalling pathway.

Docking mainly involves selection of a targeted protein design, the compound of interest or a database compound of interest or a database comprising existent or virtual compound for the docking process and a computational foundation that enable the appropriate docking and scoring methods to be implemented [3]. In a docking procedure the targeted protein is generally considered to be rigid i.e. immobile, whereas ligand is flexible. [3]

The docking procedure begins with the selection of targeted protein from the RCSB protein data bank and downloading it in the pdb format following to which using bio via discovery studio the protein and ligand files are prepared separately.

To prepare the target protein the inbuilt ligand is then deleted along with the undesired protein strands not associated with the active site of the protein. Similarly for the ligand the linked protein backbone is deleted along with the water molecules, adding the polar hydrogens and finally adding kolmann charges.

After preparing the protein both the pdb files are converted to pdbqt using Autodock Vina and is saved all in one single folder along with the run file and configuration file having the details of the coordinated of the active site. Finally using the command prompt the code for docking is run. Once the docking process is completed the docking score appears in format of decreasing order or the binding energy with respect to the different docking poses and the best docking

pose being the top score having the most negative value of binding energy the most efficient configuration for docking.

The purpose of the scoring function is to delineate the correct poses from incorrect poses, or binders from inactive compounds in a reasonable computation time. [4] Docking is one of the most widely used computational screening methods of all. This predicts the binding affinity of a complex between a receptor and a ligand. the docking process typically involves generating an ensemble of possible binding poses and their ranking by means of certain scoring function [5]. Binding affinity refers to the strength of the interaction between a protein and a ligand like small molecule or another protein and is typically expressed in units of dissociation constant (Kd) or binding constant (Kb). It is mainly influenced by shape complementary, Electrostatic interactions, hydrophobic interactions, hydrogen bonding and entropic effect.

5.1.2 Different Softwares used in molecular docking:

a) **AutoDock Vina:** It is a software tool used for molecular docking, a process in computational chemistry and biology that predicts the preferred orientation of one molecule to another when bound to each other. It is widely used mainly for drug discovery by identifying potential drug candidates by simulating how they interact with target proteins it is also used for Protein-ligand docking, studying protein-ligand interactions, crucial for understanding biological processes and designing drugs. Virtual screening Filtering large compound libraries to identify potential binders to a target protein. It is mainly known for its high accuracy, fast docking calculations easy to use interface etc.

b) **AutoDock:** AutoDock is a molecular docking software suite developed by the Scripps Research Institute. It's a widely used tool for predicting the binding mode and affinity of small molecules to proteins. The suite includes:

1. AutoDock: The original docking program, which uses a Monte Carlo simulated annealing algorithm to search for optimal binding poses.
2. AutoGrid: Prepares the grid maps necessary for docking.
3. AutoDockTools (ADT): A graphical user interface for preparing input files, running the docking program, and analyzing results.

c) **Schrodinger Software:** Schrödinger is a leading software company in computational chemistry and biology, providing innovative solutions for drug discovery, materials science, and life sciences research. Their software suite includes:

1. Maestro: A molecular modeling and simulation platform for drug discovery and materials science.
2. Glide: A molecular docking and scoring tool for predicting protein-ligand interactions.
3. Prime: A protein structure prediction and optimization tool.
4. Jaguar: A quantum mechanics and molecular mechanics simulation software.
5. Desmond: A high-performance molecular dynamics simulation software.

d) **DOCK:** DOCK (Docking Orienting and Scoring) is a molecular docking software tool used for predicting the binding orientation and affinity of small molecules to proteins. Developed at the University of California, San Francisco (UCSF), DOCK is widely used in:

1. Drug discovery: Identifying potential drug candidates
2. Lead optimization: Refining lead compounds for better affinity and specificity
3. Protein-ligand interaction studies: Understanding biological processes and designing drugs

e) **GOLD:** GOLD (Genetic Optimization for Ligand Docking) is a molecular docking software tool developed by the Cambridge Crystallographic Data Centre (CCDC). GOLD is widely used for:

1. Drug discovery: Identifying potential drug candidates
2. Lead optimization: Refining lead compounds for better affinity and specificity
3. Protein-ligand interaction studies: Understanding biological processes anddesigning drugs.

- Key features of GOLD include:

1. Genetic algorithm-based docking engine for efficient searching
2. Flexible ligand docking with optional protein flexibility
3. Scoring functions for affinity prediction, including GoldScore and ChemScore
4. Support for multiple ligand and protein formats
5. High-performance computing capabilities

f) **PyRx:** PyRx is an open-source software tool for molecular docking, specifically designed for:

1. Virtual screening: Identifying potential drug candidates from large libraries
2. Lead optimization: Refining lead compounds for better affinity and specificity

3. Protein-ligand interaction studies: Understanding biological processes and designing drugs

- PyRx features:

1. User-friendly interface for easy setup and execution
2. Support for multiple docking software, including AutoDock Vina, AutoDock 4, and GOLD
3. Integration with external tools for ligand preparation, protein preparation, and results analysis
4. High-performance computing capabilities for fast docking calculations
5. Compatibility with various operating systems, including Windows, macOS, and Linux

5.1.3 *In-silico* studies for inflammatory conditions

Inflammation is a complex process characterized by the contribution of various mediators, including prostaglandins and nitric oxide (NO). Cyclooxygenase (COX) is one of the main enzymes involved in the metabolism of arachidonic acid, catalysing the synthesis of prostaglandins and thromboxane. COX exists in two isoforms: COX-1 is a ubiquitously and constitutively expressed isoform that is postulated to have housekeeping functions; COX-2 is an inducible isoform that has been implicated in inflammatory responses and the regulation of cell growth and differentiation. Specifically, COX-2 is thought to be the primary generator of the prostanoids that contribute to inflammation, acting in both the inflammation initiation and resolution phases. However, several studies have shown that prostanoids formed via COX-1 are also involved in inflammation processes. [1]

Inflammation is a multifactorial process. It reflects the response of the organism to various stimuli and is related to many disorders such as arthritis, asthma and psoriasis which require prolonged or repeated treatment. Cyclooxygenase (COX) and lipoxygenase (LOX) produce two groups of arachidonic acid metabolites, prostaglandins (COX products) and leukotrienes (LOX products), that play a key role in inflammation. The classical nonsteroidal anti-inflammatory drugs (NSAIDs) act via the inhibition of the COX-1 isoenzyme or the combined inhibition of COX-1 and COX-2 isoenzymes. For example, aspirin is a COX-1 selective inhibitor, whereas indomethacin and naproxen are COX-1/COX-2 inhibitors. Because COX-1 is mainly responsible for mucus formation in the gastrointestinal (GI) tract, COX-1 inhibition

is implicated for inducing GI irritation, the main undesired side effect of such agents [1]. Another side effect, mild bleeding diathesis also results from the selective inhibition of the COX-1 catalyzed synthesis of the platelet aggregation factor, thromboxane A2.

There are several inflammatory mediators to make reaction during inflammation. Among these, tumour necrosis factor (TNF-α) and interleukins (IL-1β and IL-6) are pro-inflammatory cytokine, inducible nitric oxide synthase (iNOS) and cyclooxygenases (COX-1 and COX-2), which increase during inflammation and cause several diseases. In this context, several anti-inflammatory phytomedicines are used for pain relief and targeting specific immune and inflammatory pathways by inhibition of TNF-α, IL-1β, IL-6, iNOS, COX-1 and COX-2. The researchers observed that synthetic drugs have potent side effects when used for the inhibition of above-mentioned target receptors for pain and inflammation. In present research scenario, researchers are showing interest for medicines from plant origin or phytomedicines to target inflammatory mediators without any adverse effects. There are several plant species used to relief pain and prevent inflammation, oxidative stress, etc. during joint disorders. Generally, *In silico* screening, protein or receptor is the main target to detect allosteric or inhibitory activity for drug action. Several compounds or ligands are derived from synthetic compounds or phytocompounds, which show favourable binding affinity and energy for the target. This may help in new and efficient drug development as a lead molecule(s). The virtual screening helps to detect large numbers of drug-like compounds, which are commercially available, computationally screened against targets to recognize the structure and function that are predicted to bind properly in an experiment. Therefore, in recent trend, development of phytomedicines, *in silico* predictions play a vital role in the drug design and discovery process for pharmaceutical research. Moreover, the prediction of pharmacokinetics, bioavailability and druglikeness for small molecules has already been established by using SwissADME online tool. The objective of the present study is to know the binding affinity and energy of established phytochemicals as flavonoids compared to synthetic medicine (Diclofenac) against four cytokine receptors (TNF-α, COX-1, COX-2 and IL-6) through molecular docking.

Few studies have comprehensively reported the structure–anti-inflammatory activity relationship of any specific flavonoid. However, several studies have considered the relationship for flavonoids in general, primarily by comparing a single flavonoid to another flavonoid in the same or a different class. The mechanism for the anti-inflammatory action of

a given flavonoid is not determined by its class but by its attached hydroxyl groups. Knowledge of a flavonoid's structure, particularly the numbers of hydroxyl groups at the A and B rings, can allow inference of its anti-inflammatory activity. Some structure–anti-inflammatory activity relationships have been proposed for flavonoids. Specifically, the presence of a double bond between C2- C3 and a hydroxyl substitution at the 5- and 7- positions on the A-ring have been supposed necessary for anti-inflammatory activity. Higher activity levels are also shown by flavonoids containing a hydroxyl group at the 3′ and 4′- positions on the B-ring.

5.1.4 Binding energy

Binding energy in molecular docking refers to the strength of the interaction between a protein and a ligand. It is a measure of how tightly the ligand binds to the protein and is an important factor in determining the efficiency of the docking process. The binding energy is typically calculated in terms of kcal/mol and can be used to compare the binding performance of different proteins and ligands. It is influenced by various factors such as hydrogen bonding patterns and hydrophobic interactions. The goal of molecular docking is to predict the binding orientation and stability of the ligand-protein complex, and the binding energy provides valuable information in this regard. The optimization algorithms used in docking, such as genetic algorithms and multi-objective approaches, can be adjusted to enhance the binding energy and improve the efficiency of drug design.

5.1.5 Rationale for the Selection of Receptors in Molecular Docking Studies for Anti-Inflammatory Activity

5.1.5.1 TNF-α

TNF-α is a pro-inflammatory cytokine produced primarily by activated macrophages, T cells, and other immune cells in response to various inflammatory stimuli. It plays a pivotal role in the pathogenesis of numerous inflammatory and autoimmune diseases, such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, and sepsis. TNF-α exerts its effects by binding to two distinct receptors on the cell surface: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2).

1. **Mediation of Inflammatory Response**: Upon binding to its receptors, TNF-α activates multiple signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and the mitogen-activated protein kinase (MAPK)

pathway. These pathways lead to the transcription of various pro-inflammatory genes, thereby amplifying the inflammatory response.

2. **Promotion of Cytokine Production**: TNF-α stimulates the production of other pro-inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6), further propagating inflammation and contributing to the clinical symptoms of inflammatory diseases.
3. **Induction of Apoptosis**: TNF-α can induce apoptosis in certain cell types, contributing to tissue damage and exacerbating inflammatory conditions.

- **Justification for Selecting TNF-α Receptor**

  Given the central role of TNF-α in inflammation, its receptor becomes an attractive target for the development of anti-inflammatory agents. The selection of the TNF-α receptor for molecular docking studies is justified by several factors:

1. **Key Regulator of Inflammation**: Targeting the TNF-α receptor allows for the modulation of a key regulatory point in the inflammatory cascade. Inhibiting the interaction between TNF-α and its receptor can effectively reduce the downstream signaling events that lead to inflammation.
2. **Clinical Relevance**: TNF-α inhibitors, such as monoclonal antibodies (e.g., infliximab, adalimumab) and soluble receptor constructs (e.g., etanercept), have already demonstrated clinical efficacy in treating inflammatory diseases. This underscores the therapeutic potential of targeting the TNF-α receptor.
3. **Specificity and Selectivity**: Molecular docking studies can help identify compounds that specifically bind to the TNF-α receptor, minimizing off-target effects and enhancing the selectivity of potential therapeutic agents.
4. **Structure-Based Drug Design**: The availability of high-resolution crystal structures of the TNF-α receptor facilitates structure-based drug design, allowing for the precise modeling of receptor-ligand interactions and the identification of key binding sites.

- **Objectives of Molecular Docking Studies**

  The primary objectives of conducting molecular docking studies on the TNF-α receptor include:

1. **Identification of Potential Inhibitors**: Screening a library of compounds to identify those that can effectively bind to the TNF-α receptor and block its interaction with TNF-α.
2. **Understanding Binding Mechanisms**: Elucidating the molecular interactions and binding mechanisms between the receptor and potential inhibitors, providing insights into the structural requirements for binding affinity and specificity.
3. **Optimization of Lead Compounds**: Using the docking results to guide the optimization of lead compounds, enhancing their efficacy and pharmacokinetic properties for potential therapeutic use.[17]

5.1.5.2 IL-6

IL-6 is a multifunctional cytokine produced by various cell types, including T cells, B cells, macrophages, fibroblasts, and endothelial cells, in response to infections, tissue injuries, and other inflammatory stimuli. It has a wide range of biological activities that contribute to both acute and chronic inflammation.

1. **Mediation of Acute Phase Response**: IL-6 is a key regulator of the acute phase response, stimulating the liver to produce acute phase proteins such as C-reactive protein (CRP) and serum amyloid A, which are markers of inflammation.
2. **Stimulation of Immune Responses**: IL-6 promotes the differentiation of B cells into antibody-producing plasma cells and the activation of T cells, enhancing the adaptive immune response.
3. **Chronic Inflammation and Autoimmune Diseases**: Persistent elevation of IL-6 levels is associated with chronic inflammatory conditions and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis. IL-6 contributes to the pathogenesis of these diseases by promoting the survival and proliferation of pathogenic T cells and inhibiting regulatory T cells.
4. **Cytokine Storm**: IL-6 plays a pivotal role in the cytokine storm observed in severe infections, such as COVID-19, where excessive cytokine production leads to widespread inflammation and tissue damage.

- **Justification for Selecting IL-6**

  The selection of IL-6 as a target for molecular docking studies in anti-inflammatory research is justified by several critical factors:

1. **Central Role in Inflammation**: IL-6 is a central mediator of inflammation and immune regulation. Targeting IL-6 can modulate a broad spectrum of inflammatory pathways, making it a potent target for therapeutic intervention.
2. **Clinical Relevance**: IL-6 inhibitors, such as tocilizumab and sarilumab, have demonstrated clinical efficacy in treating inflammatory and autoimmune diseases. This highlights the therapeutic potential of targeting IL-6.
3. **Specificity and Selectivity**: Molecular docking allows for the identification of compounds that specifically bind to IL-6, potentially leading to selective inhibitors that minimize off-target effects.
4. **Availability of Structural Data**: High-resolution structures of IL-6 and its receptor complexes are available, facilitating detailed modeling of receptor-ligand interactions and the design of novel inhibitors.[17]

- **Objectives of Molecular Docking Studies**

The primary objectives of conducting molecular docking studies on IL-6 include:

1. **Identification of Potential Inhibitors**: Screening compound libraries to identify molecules that can effectively bind to IL-6 and inhibit its interaction with its receptor, thereby blocking its pro-inflammatory activity.
2. **Understanding Binding Mechanisms**: Elucidating the molecular interactions and binding mechanisms between IL-6 and potential inhibitors, providing insights into the structural features necessary for high binding affinity and specificity.
3. **Optimization of Lead Compounds**: Using docking results to guide the optimization of lead compounds, enhancing their therapeutic efficacy and pharmacokinetic properties for potential clinical use.

5.1.5.3 COX-1 and COX-2[19]

Cyclooxygenases, commonly known as COX enzymes, are key enzymes in the biosynthesis of prostaglandins, which are lipid compounds that play diverse roles in inflammation, pain, and fever. There are two main isoforms of cyclooxygenase: COX-1 and COX-2.

A) **COX-1**:

- **Constitutive Expression**: COX-1 is constitutively expressed in most tissues and is involved in maintaining normal physiological functions, including the protection of the gastric mucosa, regulation of blood flow in the kidneys, and platelet aggregation.
- **Housekeeping Functions**: It is often referred to as a "housekeeping" enzyme because it regulates homeostatic functions in the body.[11]

B) **COX-2**:

- **Inducible Expression**: COX-2 is an inducible enzyme, primarily expressed in response to inflammatory stimuli, such as cytokines, growth factors, and bacterial lipopolysaccharides (LPS).
- **Role in Inflammation**: COX-2 is responsible for the production of pro-inflammatory prostaglandins, which mediate inflammation, pain, and fever.

- **Justification for Selecting COX-1 and COX-2**

  The selection of COX-1 and COX-2 as targets for molecular docking studies in the context of anti-inflammatory research is justified by several critical factors:

- **Central Role in Inflammatory Pathways**:
  - **COX-2**: As a major enzyme responsible for the production of pro-inflammatory prostaglandins, COX-2 is directly involved in mediating the symptoms of inflammation, making it an ideal target for anti-inflammatory drugs.
  - **COX-1**: While COX-1 is not primarily involved in inflammation, it is crucial to study it to avoid unwanted side effects, such as gastrointestinal irritation and bleeding, which are associated with the inhibition of COX-1.

- **Clinical Relevance**:
  - Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, and naproxen inhibit both COX-1 and COX-2 to alleviate inflammation and pain. However, selective COX-2 inhibitors (e.g., celecoxib) have been developed to reduce inflammation while minimizing gastrointestinal side effects associated with COX-1 inhibition.
  - Understanding the differential inhibition of COX-1 and COX-2 is essential for developing safer and more effective anti-inflammatory drugs.

- **Specificity and Selectivity**:
  - Molecular docking allows for the identification of compounds that selectively inhibit COX-2 over COX-1, enhancing therapeutic efficacy while reducing side effects.
  - Structure-based drug design facilitated by molecular docking can pinpoint the precise interactions required for selective inhibition.

- **Availability of Structural Data**:
  - High-resolution crystal structures of both COX-1 and COX-2 are available, providing detailed insights into their active sites and aiding in the rational design of selective inhibitors.

- **Objectives of Molecular Docking Studies**

  The primary objectives of conducting molecular docking studies on COX-1 and COX-2 include:

1. **Identification of Potential Inhibitors**:
   - Screening compound libraries to find molecules that effectively bind to COX-1 and COX-2, with an emphasis on identifying selective COX-2 inhibitors.
2. **Understanding Binding Mechanisms**:
   - Elucidating the molecular interactions between the COX enzymes and potential inhibitors to understand the structural basis for binding affinity and selectivity.
3. **Optimization of Lead Compounds**:
   - Using docking results to guide the optimization of lead compounds, improving their efficacy, selectivity, and pharmacokinetic properties for potential clinical use.

   COX-1 and COX-2 are expressed under normal conditions in the human body and have physiological and immunological activity in some tissues such as cytoprotection of gastrointestinal tissues and platelet aggregation by COX-1, fundamental function of the brain by COX-2 and immunoreactivities in brain by both COX-1 and COX-2. However, beside vital activity in the brain, COX-2 plays an important role in the inflammation scenario in response to cytokines and pro-inflammatory molecules.[11]

   COXs are bi-functional heme proteins that catalyse the conversion of AA into prostaglandin precursors in two steps – cyclo-oxygenation and peroxidation, which occur separately in two distinct regions of the protein. Crystallography studies indicated that the target site for NSAIDs (Non- Steroidal Anti-Inflammatory Drugs) is the cyclo-oxygenation pocket

Prolonged use of NSAIDs leads to gastrointestinal and renal complications.[5] The discovery of COX-2, an inducible isoform of COX, and studies suggesting the role of COX-1 products as key players in housekeeping functions (including gastrointestinal, vascular, and renal homeostasis) motivated the scientific community to draw a correlation between the levels of COX-2 products and inflammation. The observation that NSAIDs are nonspecific and inhibit both the isoforms, led to a spurt in the interest to identify selective COX-2 inhibitors (COXIBs), that could eliminate the side effects caused by the usage of NSAIDs. Additionally, it was observed that prostaglandins act as both pro- or anti-inflammatory mediators based on the tissue/ organ of action. This contradiction is not limited to prostaglandins as the studies suggested that, COX-2 is constitutively expressed and is responsible for homeostasis in certain regions (ex: central nervous system, kidneys), where COX-1 is the induced isoform. For example, in the case of the central nervous system, it is observed that the COX-1 products are responsible for modulating inflammation suggesting that there is a need to develop specific COX-1 inhibitors to treat neurodegenerative diseases. Hence, the focus has shifted back to non-specific inhibitors and efforts are being made to develop NSAIDs with an innate capability to overcome/ prevent gastrointestinal complications. This is being achieved by modifying the NSAID backbone with various functional groups to develop chimeric NSAIDs, including NO releasing, H_2S-releasing, and antioxidant NSAIDs. Thus, there is a need for inhibitors that could interact with multiple targets (COX-1, COX-2, and LOX) and modulate their products while remaining GI safe. [18]

5.2 Methodology

5.2.1 Retrival of phytoconstituents of all three plants

The list of chemical constituents of plants was obtained through literature survey. Majority of phytoconstituents were obtained from IMPPAT (Indian Medicinal Plants, Phytochemistry and Therapeutics)[23,24]

5.2.2 Preparation of Ligands

Smiles format of all ligands were obtained from PubCHEM, Drug bank and ZINC database. These ligands were subjected to Frog: a FRee Online druG 3D conformation generator. The

process consists of a series of steps that perform conversions, apply corrections to the structures, generate variations on the structures, eliminate unwanted structures, and optimize the structures. Many of the steps are optional, and are controlled by selecting options in the panel or specifying command-line options. The steps involved were as follows: structure format was converted, Structures were selected, hydrogen atoms were added, unwanted molecules were removed, charged groups were neutralized, ionization states were generated, tautomers were generated, Structures were filtered, alternative chiralities were generated, low-energy ring conformations were generated and geometry of the ligands were optimized. Ligand structure with minimum potential energy and similar chirality to that of selected ligand IUPAC name was selected for the docking experiment. All ligands were obtained in sdf format.

5.2.3 Retrieval of Target Sequence

The FASTA format of key targets involved in inflammation were retrieved from the RCSB Protein Data Bank. The details of their accession numbers and resolution are mentioned in Table 5.1. Amongst the different PDB IDs available at the protein databank site, the files having lowest resolution and Homo sapiens as the source was given preference. Also, the method by which structure was obtained was selected as X-ray diffraction method. The complexes bound to the receptor were removed using SPDBV, and the non-essential water molecule was removed and polar hydrogen was added and the already prepared receptor was saved in PDB format.

Table 5.1 Details of Target receptors used for molecular docking

Protein (Target)	PDB Accession number	Source	Resolution A^0	Method by which structure was obtained
Interleukin 6	1ALU	Homo sapiens	1.9	X-ray diffraction
Tumor Necrosis Factor- Alpha	1TNF	Homo sapiens	2.6	X-ray diffraction
COX-2	3LN1	Mus musculus	2.4	X-ray diffraction
COX-1	3N8Y	Ovis aries	2.6	X-ray diffraction

5.2.4 Molecular docking with software:

Molecular docking was done using PyRx and AutoDock vina. PyRx is a Virtual Screening software for Computational Drug Discovery that can be used to screen libraries of compounds against potential drug targets. Autodock vina is an in-built feature of PyRx. All ligands are loaded in sdf format with the help of OpenBable. These ligands are then minimized and converted into pdbqt format as a part of preparation for docking. Macromolecule (target protein) is loaded in PyRx in pdb format. This protein was prepared for docking by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking was carried out using AutoDock vina as an in-built feature of PyRx on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.

5.3 Results and Discussion

5.3.1 Result analysis and Complex interaction visualization

Results are obtained in the form of binding affinity of ligands towards the protein. Ligands having highest binding affinity are sought to be most interacting ligand. Complex of ligand-protein was visualized using PyMoL and residual interactions were obtained from LigPLOT.

5.3.2 Docking studies

The key parameter created as a result of molecular docking is binding energy. It provides information on the intensity and affinity of the ligand-receptor interaction. The weaker the contact, the higher the binding energy, and vice versa. As a result, we looked for the ligand with the lower binding energy, thus conferring good affinity towards the target, among the test compounds, during docking process. The phytocompounds that exhibited binding affinity towards more than one targets, but only the one's exhibiting least docking score and least binding energy was considered as the suitable ligand-target interaction. In this study, the phytocompounds that demonstrated good measurable binding affinities for the target residues was considered. The binding affinities were indicative of the ligand's contribution to and flexibility for the target.

5.3.2.1 3LN1 (COX-2)

Identification of active sites is a pre-requisite requirement for appropriate molecular docking. Active sites were identified through Pymol visualization. During molecular docking a grid is to be selected which represents the area where ligands will try to interact with the protein. Center Grid box was selected based on co-crystallized ligand celecoxib, using x: 32.913, y: -25.887, z: -5.511, and the number of points in all dimensions x, y, z were considered 58x68x66 Å, and the grid spacing was selected as 0. 96 Å. The binding affinities of all the phytoconstituents is given in table no. 5.2

Table 5.2 Binding affinity of constituents against COX-2

Ligand	**Binding affinity ((kcal/mol)**
Artemiseole	-4.91
Dihydromyrcene	-3.99
AC1NSUBD	-4.67
Anisotine	**-7.15**
Calotropagenin	-5.15
Hexopyranose	-2.83
Benzoyllineolone	-4.65
Isosteviol	**-6.55**
Peganine	-5.43
Daucosterol	-3.62
Vasicinone	-5.36
Phytosterols	-5.53
Vasicinol	-5.43
Phytosterols	-5.19
Syriogenin	-6.08
Phytosterols	-4.77
Vasicinolone	-5.24
Betaine	-2.59
Vasicol	-4
α-Amyrin	**-9.22**

β-Amyrin	**-7.08**
Butanoic acid	-2.93
Octadeca-9,12-dienoic acid	-2.83
Proceragenin A	-6.08
Oleic acid	-1.73
Lignoceric acid	-0.56
Caryophyllene oxide	-5.88
2',4'-dihydroxychalcone 4'-2 glucoside	-3.84
Calactinic acid	-4.35
Santolina epoxide	-4.26
Octadecanoic acid	-1.95
Pentyl phenyl acetate	-3.68
Palmitic acid	-2.52
Arachidic acid	-2.21
Docosanoic acid	-4.56
Hexacosanoic acid	0.06
Diclofenac (Standard)	**-9.8**

From the above table it can be seen that α-amyrin, β- amyrin of *Calotropis procera* and Anisotine of *Adhatoda Vasaka* had the highest binding affinity of -9.22, -7.08 and -7.15 kcal/mol towards the receptor respectively. The phytoconstituents of *Rosa indica* were not able to show good binding affinity towards the receptor however, Isosteviol had fair binding affinity of -6.55 kcal/mol. Figure 5.1,5.2 and 5.3 shows the interaction of α-amyrin, Anisotine and Isosteviol with COX-2 receptor respectively.

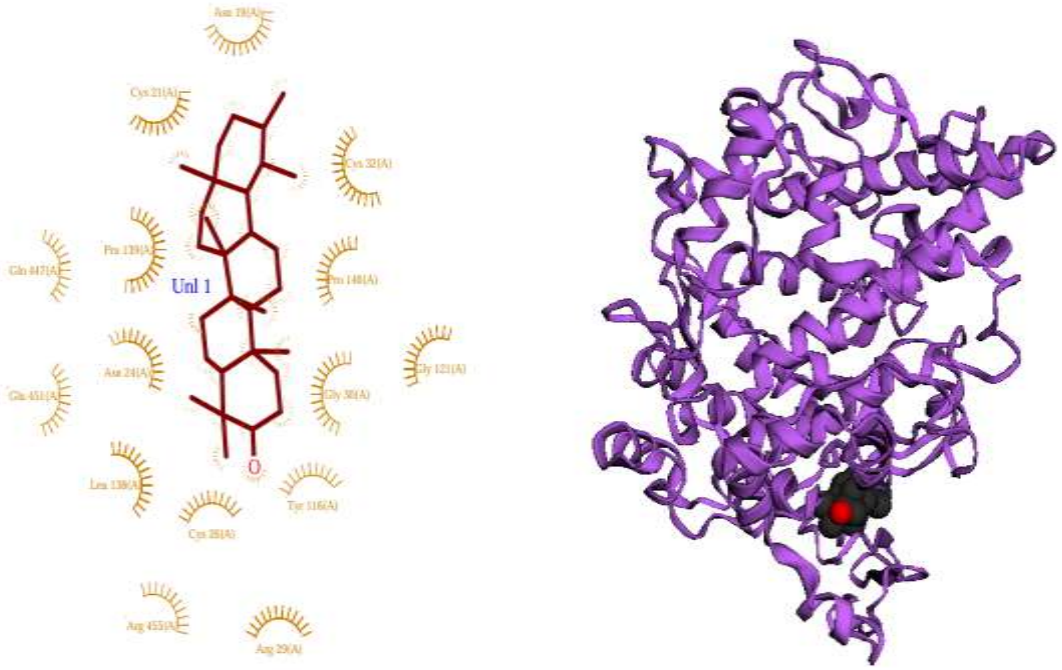

Figure 5.1 The 2D and 3D interaction of the α-amyrin with COX-2

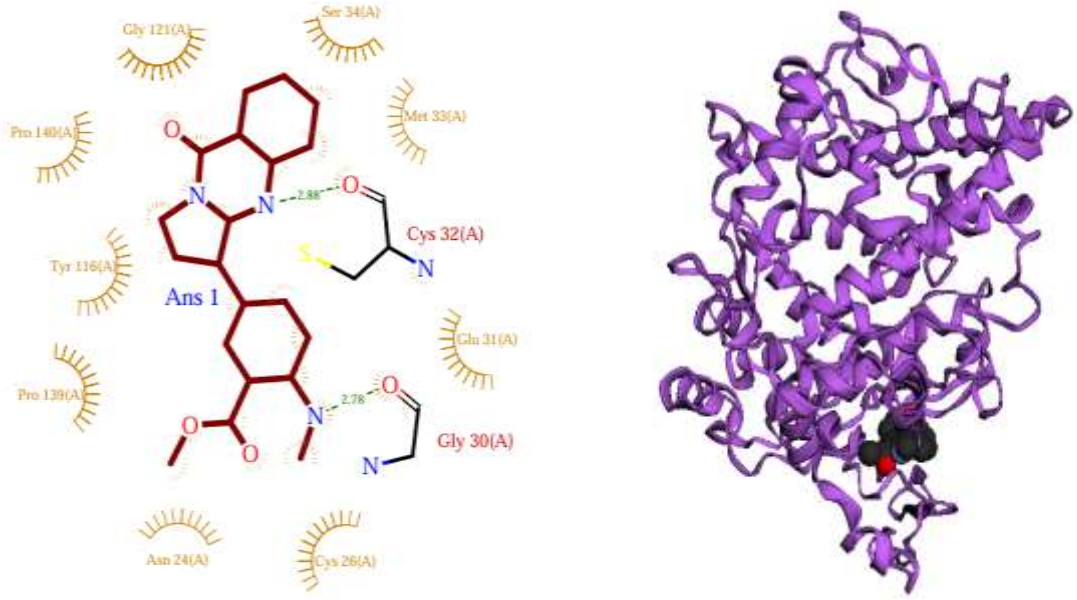

Figure 5.2 The 2D and 3D interaction of the Anisotine with COX-2

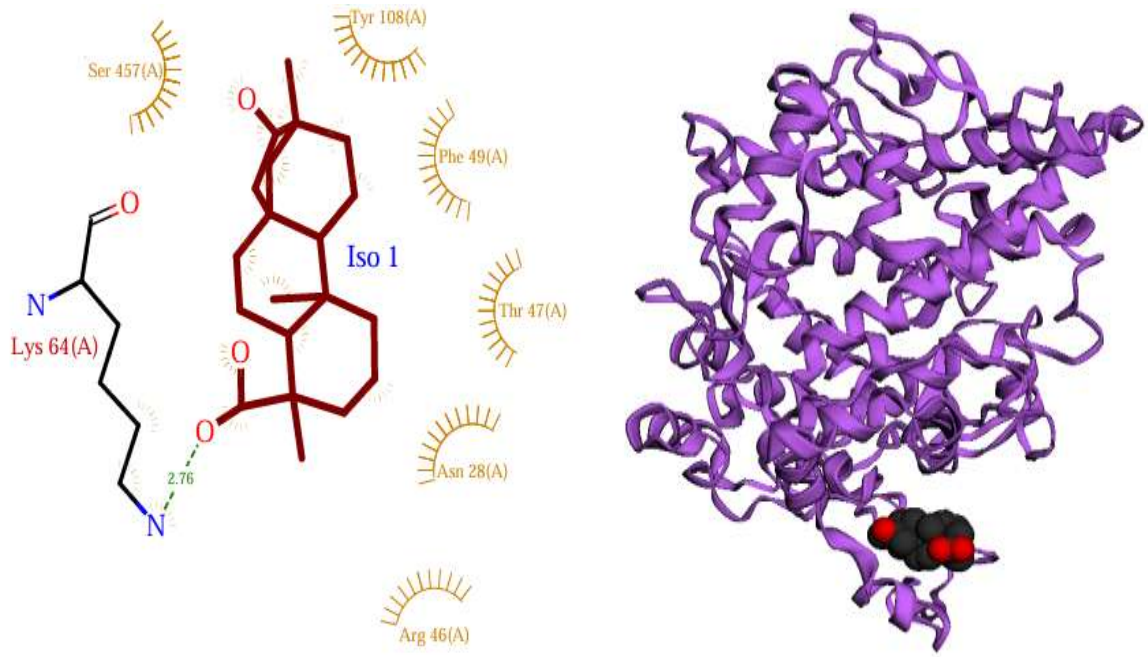

Figure 5.3 The 2D and 3D interaction of the Isosteviol with COX-2

From the above figure it can be seen that α-amyrin had the highest number of interactions with COX-2 receptor. Various hydrophobic interactions, including π – π stacking, π – cation, and π – σ interactions were responsible for the highest binding affinity of α-amyrin. Anisotine showed the second highest binding affinity owing to major role of two hydrogen bonds formed with GLY 30 and CYS 32 residues of COX-2 protein along with the other non-bonded interactions. Isosteviol showed one hydrogen bond interaction with LYS 64 residue of COX-2 with a considerable reduced non-bonded interactions leading to a fair binding affinity.

5.3.2.2 1ALU (Interleukin 6)

Identification of active sites is a pre-requisite requirement for appropriate molecular docking. Active sites were identified through Pymol visualization. During molecular docking a grid is to be selected which represents the area where ligands will try to interact with the protein. Center Grid box was selected using x: -0.233, y: -20.308, z: 9.829, and the number of points in all dimensions x, y, z were considered 44x52x40 Å, and the grid spacing was selected as 0. 94 Å. The binding affinities of all the phytoconstituents is given in Table no. 5.3

Table 5.3 Binding affinity of constituents against IL-6

Ligand	Binding affinity (kcal/mol)
Betaine	-2.36
AC1NSUBD	-4.51
Anisotine	**-5.8**
Phytosterols	-5.24
Arachidic acid	-0.98
2',4'-dihydroxychalcone 4' glucoside	-1.93
Docosanoic acid	-0.46
Daucosterol	-3.52
Peganine	-4.05
Octadeca-9,12-dienoic acid	-1.86
Oleic acid	-1.42
Vasicinol	-4.3
Lignoceric acid	0.55
Vasicinone	-4.02
Vasicinolone	-4.38
Hexacosanoic acid	1.28
Vasicol	-2.7
α-Amyrin	-5.98
β-Amyrin	**-6.43**
Proceragenin A	-5.28
Phytosterols	-5.06
Hexopyranose	-1.76
Calotropagenin	-4.09
Benzoyllineolone	-4.34
Calactinic acid	-3.68
Syriogenin	-4.62
Artemiseole	-3.64
Isosteviol	**-5.81**
Butanoic acid	-3.71

Caryophyllene oxide	-4.65
Dihydromyrcene	-2.88
Santolina epoxide	-2.9
Palmitic acid	-1.7
Pentyl phenyl acetate	-2.16
Octadecanoic acid	-1.68
Diclofenac (Standard)	**-5.4**

From the above table it can be seen that β- amyrin of *Calotropis procera*, Anisotine of *Adhatoda Vasaka* and Isosteviol of *Rosa indica* had the highest binding affinity of -6.43, -5.81 and -5.8 kcal/mol towards IL-6 respectively which is higher binding affinity than standard drug (Diclofenac- -5.4 kcal/mol). The standard Diclofenac had binding affinity lower than these three phytoconstituents. Figure 5.4, 5.5 and 5.6 shows the interaction of β-amyrin, Anisotine and Isosteviol with IL-6 receptor.

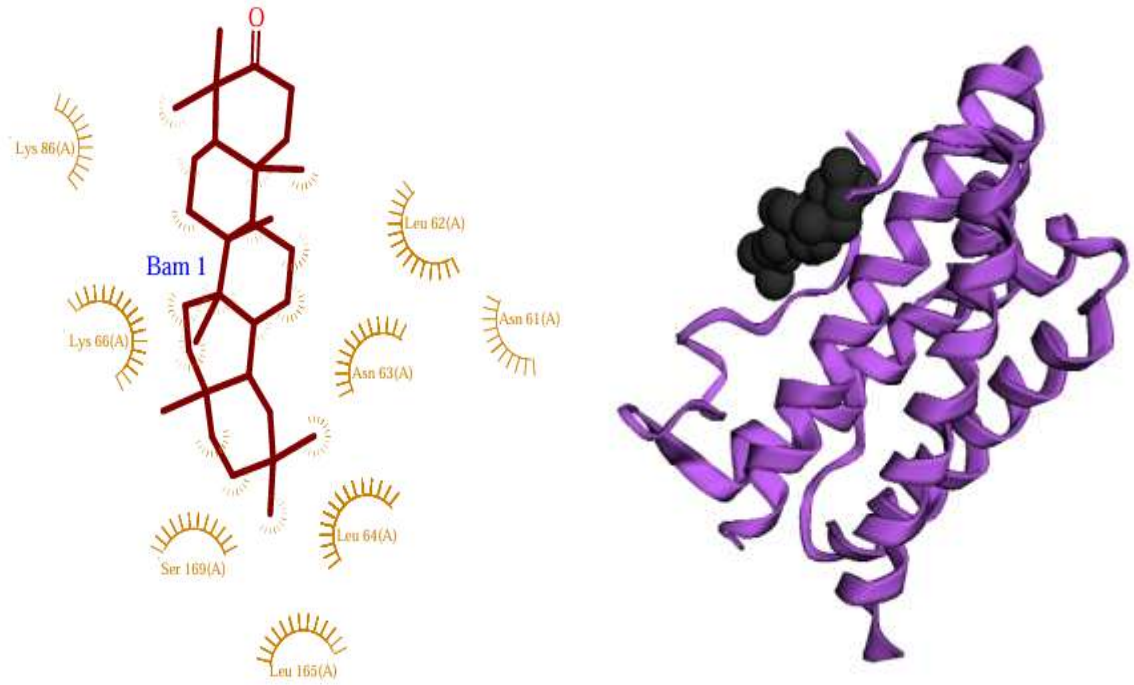

Figure 5.4 The 2D and 3D interaction of the β-amyrin with IL-6

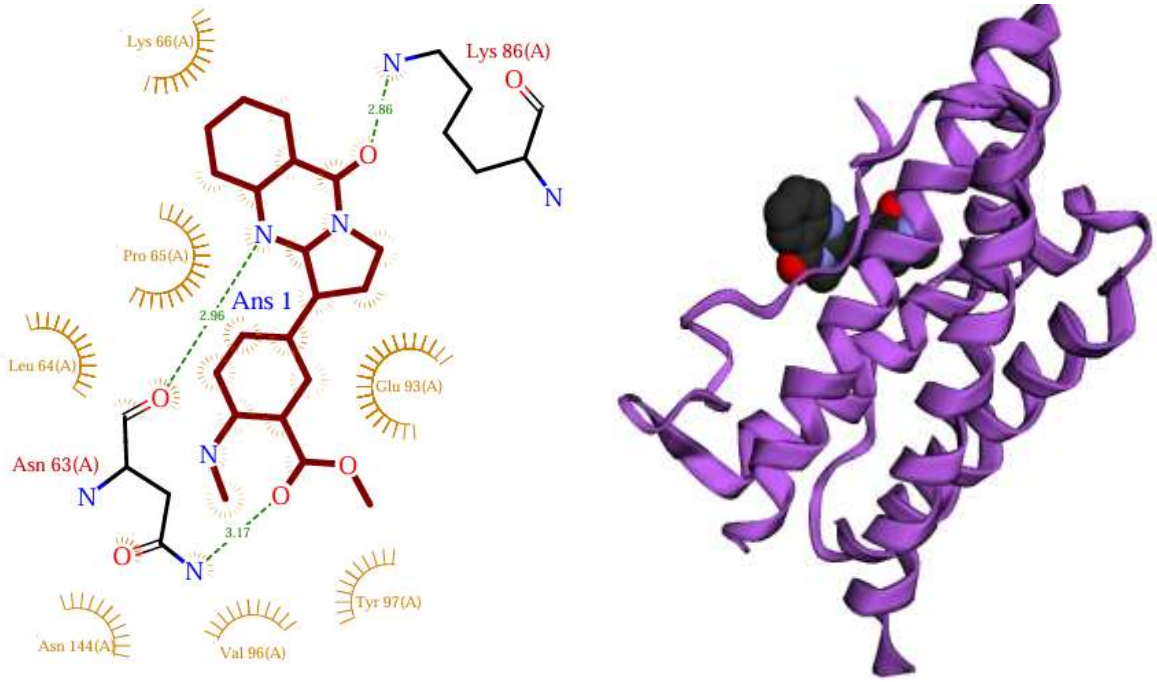

Figure 5.5 The 2D and 3D interaction of the Anisotine with IL-6

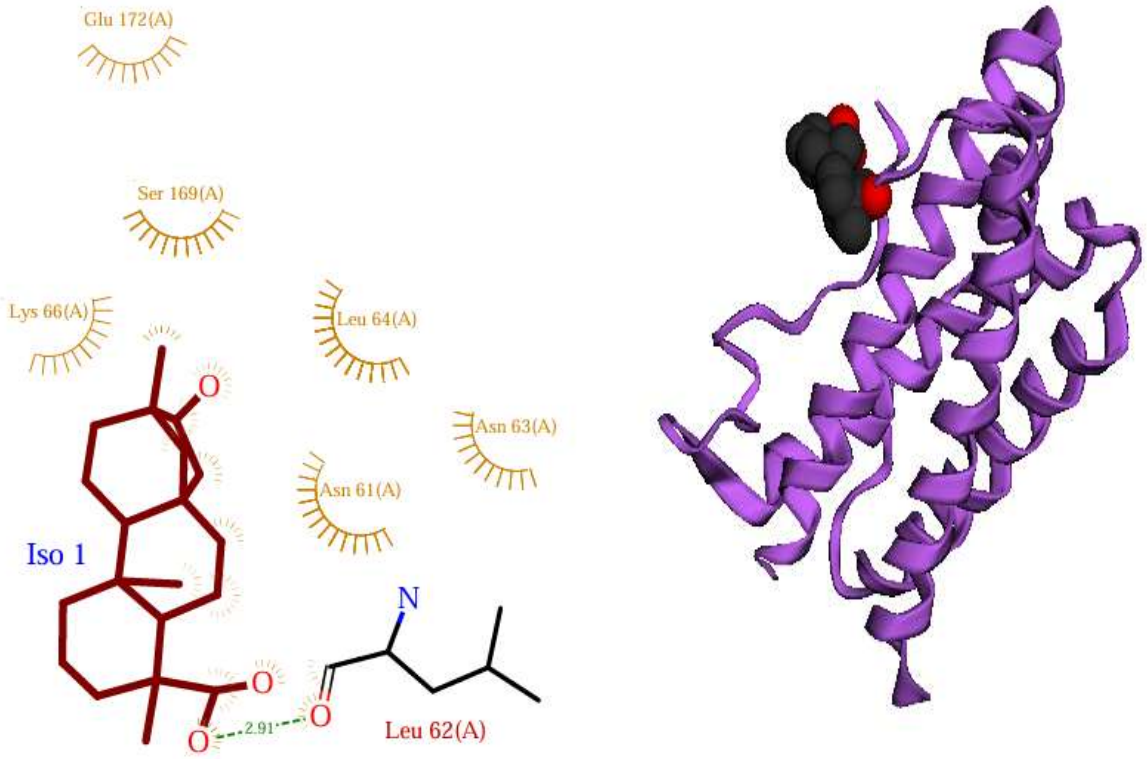

Figure 5.6 The 2D and 3D interaction of the Isosteviol with IL-6

From the above figure it can be seen that β-amyrin had the highest number of interactions with IL-6 receptor. Various hydrophobic interactions, including π – π stacking, π – cation, and π – σ interactions were responsible for the highest binding affinity of β-amyrin. Anisotine showed the second highest binding affinity owing to major role of three hydrogen bonds formed with ASN 63 and LYS 86 residues of IL-6 protein along with the other non-bonded interactions. Isosteviol showed one hydrogen bond interaction with LEU 62 (A) residue of IL-6 with a considerable reduced non-bonded interactions leading to a fair binding affinity.

5.3.2.3 3N8Y (COX-1)

Identification of active sites is a pre-requisite requirement for appropriate molecular docking. Active sites were identified through Pymol visualization. During molecular docking a grid is to be selected which represents the area where ligands will try to interact with the protein. Center Grid box was selected based on co-crystallized ligand Diclofenac, using x: 30.330, y: -47.892, z: 1.157, and the number of points in all dimensions x, y, z were considered 78x64x62 Å, and the grid spacing was selected as 0. 94 Å. The binding affinities of all the phytoconstituents is given in Table no. 5.4

Table 5.4 Binding affinity of constituents against COX-1

Ligand	Binding affinity (kcal/mol)
Betaine	-2.46
AC1NSUBD	-4.37
Anisotine	-4.38
Phytosterols	-4.96
Arachidic acid	-1.56
2',4'-dihydroxychalcone 4' glucoside	-2.45
Daucosterol	-2.72
Docosanoic acid	-1.5
Peganine	-5.12
Cctadeca-9,12-dienoic acid	-1.95
Oleic acid	-1.81
Lignoceric acid	-0.01
Hexacosanoic acid	0.68

Vasicinol	**-5.71**
VASICINONE	-5.18
Vasicinolone	-5.25
Vasicol	-3.8
α-Amyrin	**-7.13**
β-Amyrin	**-7.36**
Proceragenin A	-6.12
Phytosterols	-5.3
Calotropagenin	-5.36
Benzoyllineolone	-4.8
Hexopyranose	-2.34
Calactinic acid	-4.99
Artemiseole	-4.84
Isosteviol	**-6.01**
Syriogenin	-5.61
Butanoic acid	-3.74
Caryophyllene oxide	-5.01
Dihydromyrcene	-4.19
Santolina epoxide	-4.4
Octadecanoic acid	-1.79
Pentyl phenyl acetate	-6.24
Palmitic acid	-2.27
Diclofenac	**-7.8**

From the above table it can be seen that β- amyrin of *Calotropis procera* and Isosteviol of *Rosa indica* had the highest binding affinity of -7.36 and -6.01 kcal/mol towards the receptor respectively. The phytoconstituents of *Adhatoda Vasaka* were not able to show good binding affinity towards the receptor however, Vasicinol had fair binding affinity of -5.71 kcal/mol. Figure 5.7, 5.8 and 5.9 shows the interaction of β-amyrin, Isosteviol and Vasicinol with COX-1 receptor.

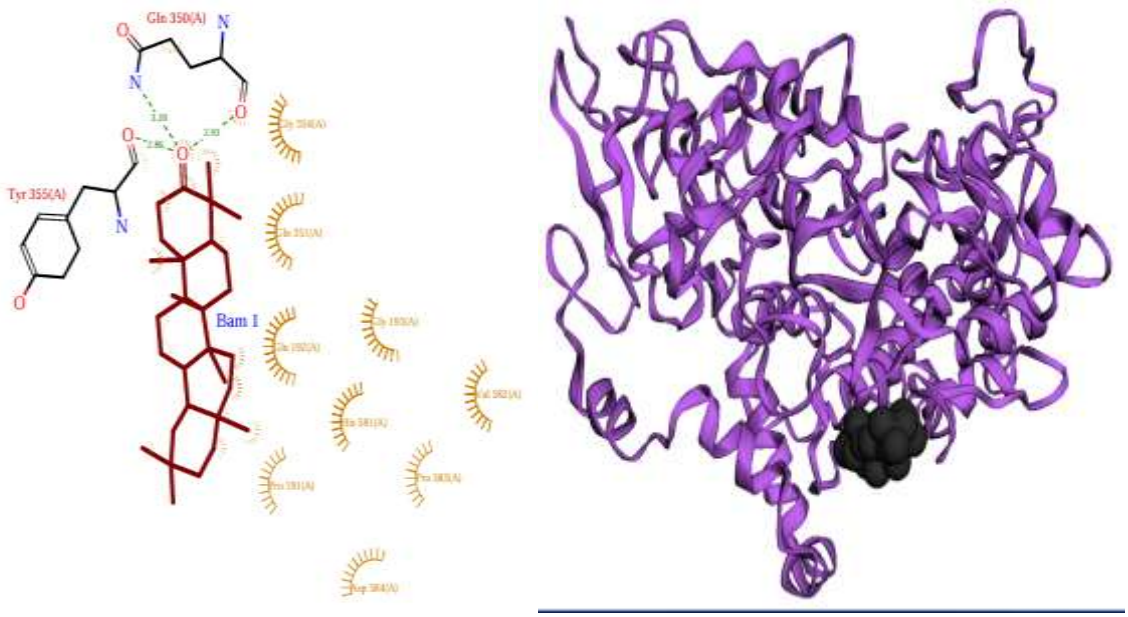

Figure 5.7 The 2D and 3D interaction of the β-amyrin with COX-1

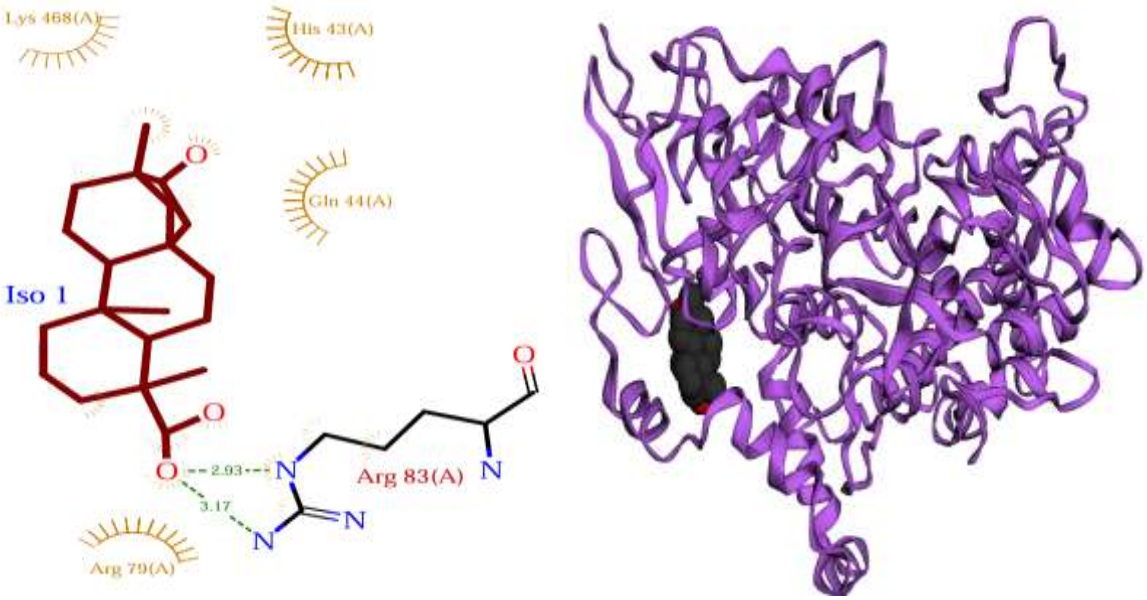

Figure 5.8 The 2D and 3D interaction of the Isosteviol with COX-1

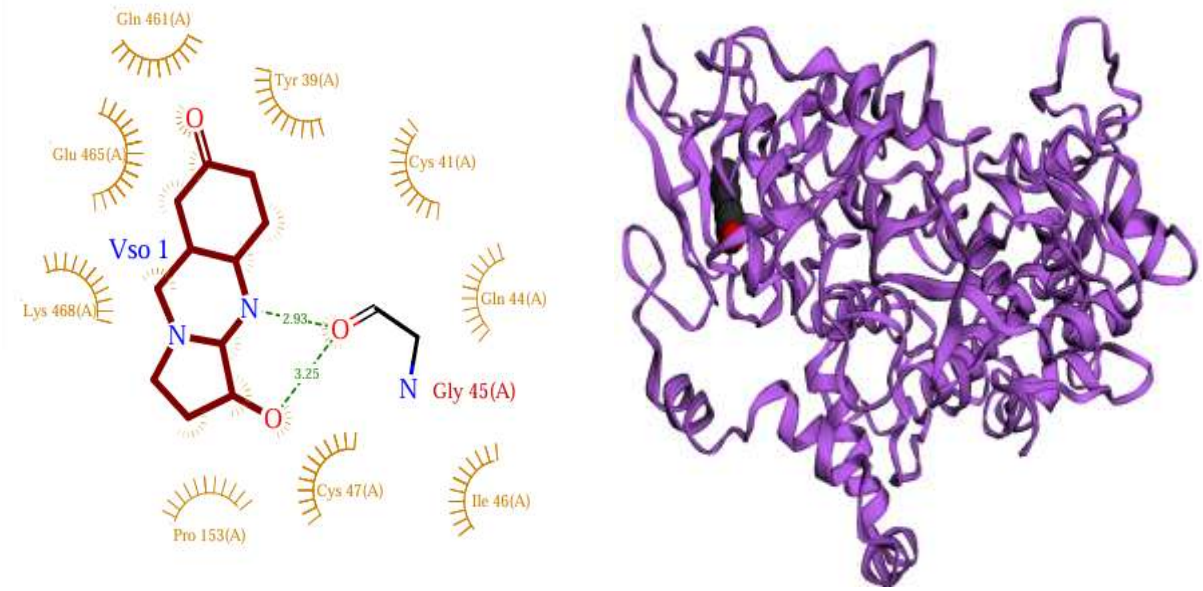

Figure 5.9 The 2D and 3D interaction of the Vasicinol with COX-1

From the above figure it can be seen that β-amyrin had the highest number of interactions with COX-1 receptor. It formed three hydrogen bond interactions GLN 350 and TYR 355 residues of the receptor along with various hydrophobic interactions, including π – π stacking, π – cation, and π – σ interactions led the highest binding affinity of β-amyrin. Isosteviol showed the second highest binding affinity owing to major role of three hydrogen bonds formed with ARG 83 residue of COX-1 protein along with the other non-bonded interactions. Vasicinol showed two hydrogen bond interaction with GLY 45 residue of COX-1 with a considerable reduced non-bonded interactions leading to a fair binding affinity.

5.3.2.4 1TNF (TNF-α)

Identification of active sites is a pre-requisite requirement for appropriate molecular docking. Active sites were identified through Pymol visualization. During molecular docking a grid is to be selected which represents the area where ligands will try to interact with the protein. Center Grid box was selected using x: 21.122, y: 48.080, z: 39.744, and the number of points in all dimensions x, y, z were considered 54x54x54 Å, and the grid spacing was selected as 0. 97 Å. The binding affinities of all the phytoconstituents is given in Table no. 5.5

Table 5.5 Binding affinity of constituents towards TNF-α

Ligand	**Binding Energy (kcal/mol)**
Artemiseole	-5.35
Dihydromyrcene	-3.48
AC1NSUBD	-6.1
Anisotine	**-5.97**
Calotropagenin	-7.41
Hexopyranose	-3.52
Benzoyllineolone	-4.38
Isosteviol	**-7.3**
Peganine	-4.66
Daucosterol	-1.86
Vasicinone	-5.09
Phytosterols	-5.93
Phytosterols	-6.5
Vasicinol	-4.94
Syriogenin	**-7.94**
Phytosterols	**-7.65**
Vasicinolone	-5.44
Vasicol	-4.43
α-Amyrin	-7
Betaine	-3.74
β-Amyrin	**-8.17**
butanoic acid	-4.66
Octadeca-9,12-dienoic acid	-2.06
Proceragenin A	-5.74
Oleic acid	-0.72
Lignoceric acid	-0.52
Caryophyllene oxide	-5.91
2',4'-dihydroxychalcone 4' glucoside	-5.13
Santolina epoxide	-3.76

Calactinic acid	-3.55
Octadecanoic acid	-1.31
Pentyl phenyl acetate	-3.93
Palmitic acid	-2.43
Arachidic acid	-1.43
Docosanoic acid	-1.29
Hexacosanoic acid	-0.77
Diclofenac	**-8.2**

From the above table it can be seen that α-amyrin, β- amyrin of *Calotropis procera* and Isosteviol of *Rosa indica* had the highest binding affinity of -8.17 and -7.3 kcal/mol towards the receptor respectively. The phytoconstituents of *Adhatoda Vasaka* were not able to show good binding affinity towards the receptor however, Anisotine had fair binding affinity of -5.97 kcal/mol. Figure 5.10, 5.11 and 5.12 shows the interaction of β-amyrin, Isosteviol and Anisotine with TNF-α receptor.

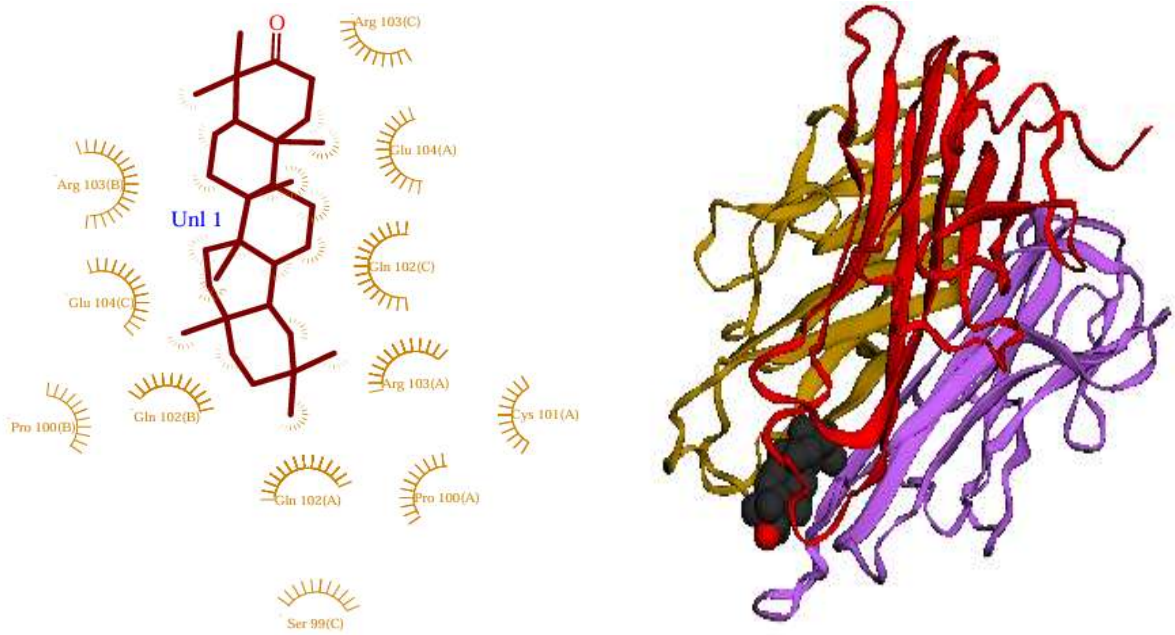

Figure 5.10 The 2D and 3D interaction of the β-amyrin with TNF-α

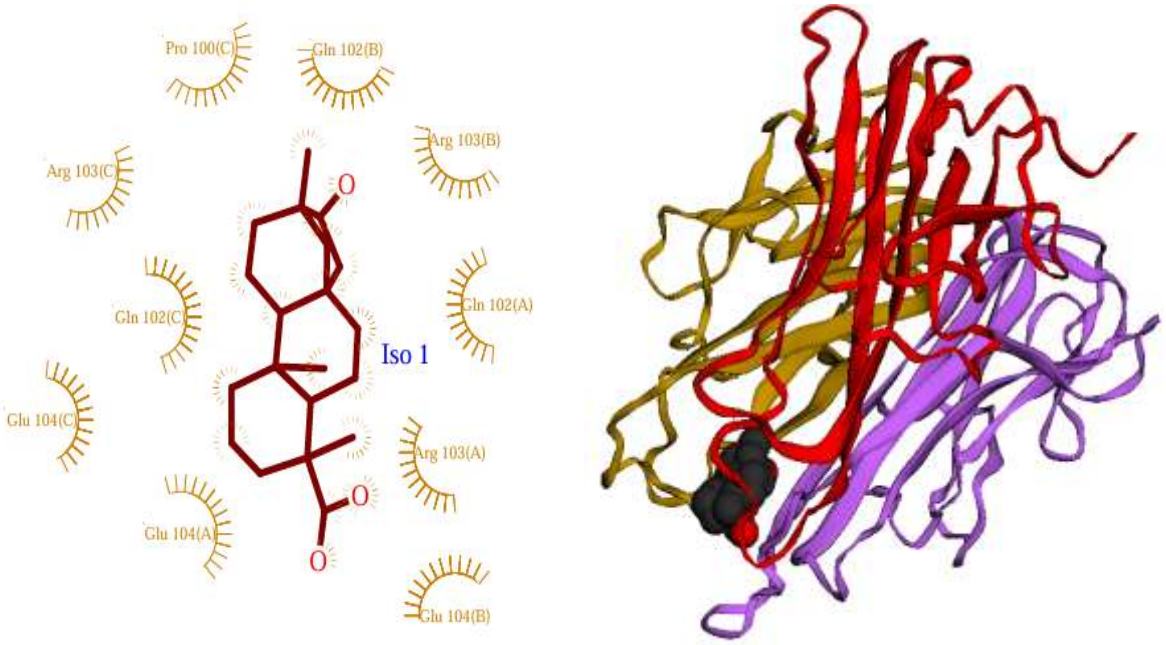

Figure 5.11 The 2D and 3D interaction of the Isosteviol with TNF-α

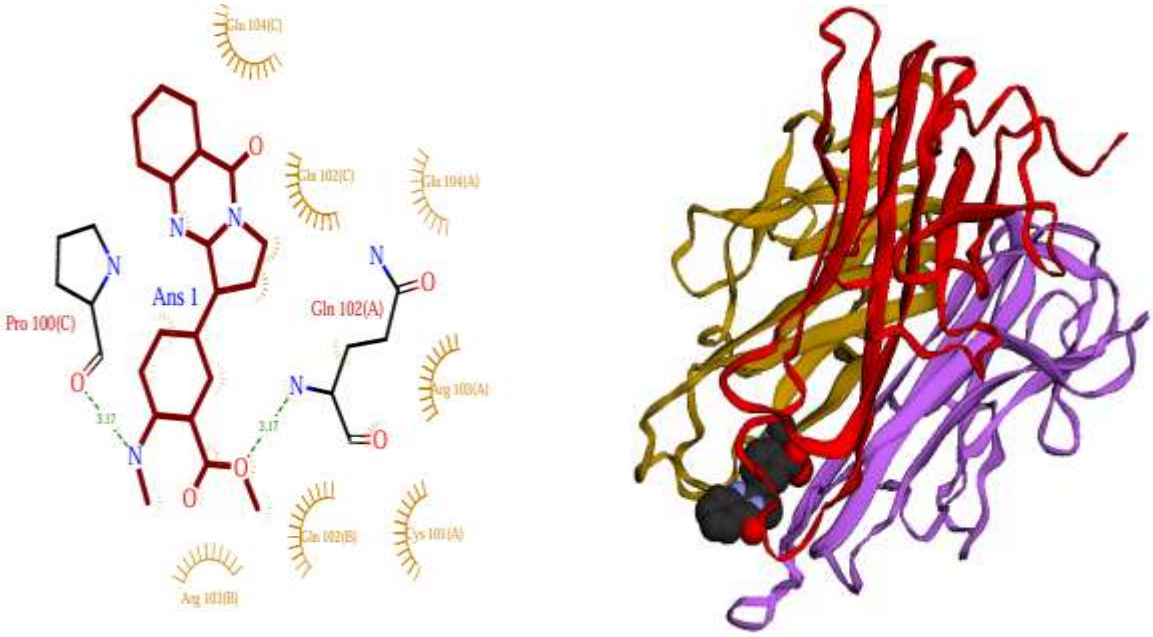

Figure 5.12 The 2D and 3D interaction of the Anisotine with TNF-α

From the above figure it can be seen that β-amyrin had the highest number of interactions with TNF-α receptor. Various hydrophobic interactions, including π – π stacking, π – cation, and π – σ interactions were responsible for the highest binding affinity of β-amyrin. Isosteviol showed high amount of non-bonded interactions. Anisotine showed two hydrogen bond interaction with

Gln 102 and Pro 100 residues of TNF-α with a considerable reduced non-bonded interactions leading to a fair binding affinity.

5.4 Conclusion of In-silico studies

Molecular docking for compounds of all three plants was performed against various receptors like COX-1, COX-2, IL-6 and TNF-α. From the results it can be concluded that TNF-α had best results in terms of maximum number of ligands showing higher binding affinity followed by COX-2 and COX-1 receptors. Molecular docking results of IL-6 were least satisfactory since most ligands showed poor binding affinity towards IL-6.

The comparison among the binding capacity of particular ligand towards various receptor lead to the conclusion of α and β-Amyrin to be the best ligands having highest binding affinity towards all kind of receptors. Other active compounds that showed relatively good binding affinity towards maximum number of receptors are Syriogenin, phytosterol, Calaptropagenin and Isosteviol. Hence it can be concluded that all three plants have components that can be responsible for potential anti-inflammatory activity.

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