Chapter 6

Identification of Inflammation-related targets of Natural Lactones using Network Pharmacology, Molecular Modeling, and *In vitro* Approaches

6 Identification of Inflammation-related Targets of Natural Lactones using Network Pharmacology, Molecular Modeling, and *In vitro* Approaches

6.1 Introduction

Natural lactones are a diverse class of secondary metabolites found in various plants and have anti-inflammatory properties. They have been used for centuries in traditional and folklore medicine to alleviate inflammation and related conditions [162]. The mechanism of action of natural lactones in inflammation is not fully understood but, they are thought to work by inhibiting the production of inflammatory mediators such as prostaglandins and cytokines [163].

Some famous examples of the ethnopharmacological use of natural sesquiterpene lactones in inflammation include the use of artemisinin (antimalarial constituent from *Artemisia annua*) in traditional Chinese medicine for their potent anti-inflammatory properties [164]. Feverfew (*Tanacetum parthenium*), a traditional herbal remedy for headaches and migraines containing parthenolide lactone as a major active constituent [20]. *Andrographis paniculata*, a medicinal herb, contains andrographolide, a sesquiterpene lactone with anti-inflammatory and immunomodulatory properties [165]. Ginkgolides and bilobalides found in the ginkgo tree (*Ginkgo biloba*) have various anti-inflammatory effects [166-168]. Cynaropicrin is a sesquiterpene lactone from *Saussurea lappa* with anti-inflammatory and anti-ulcer properties [169].

Inflammation is an evolutionary conserved adaptive immune response initiated by the host system to restore cellular homeostasis against bacteria, viruses, toxins, infections, and other foreign particles by removing harmful agents and aiding in the repair of tissues [170]. It is a complex process with various mechanisms involved in its initiation, regulation, and resolution. A typical inflammatory reaction involves a controlled increase in inflammation when there is a threat, followed by resolution once the threat is gone

[171, 172]. However, factors such as social, psychological, environmental, and biological conditions can hinder the resolution of acute inflammation that can lead to a state of systemic inflammation characterized by the activation of different immune elements compared to those involved in acute responses. When inflammation shifts from acute to chronic, it can disrupt immune tolerance and cause significant changes in tissues, organs, and normal cellular functions, ultimately affecting immune responses [170]. Several key regulators play a key role in the mediation of the inflammatory response, determining proinflammatory molecule expression. Inflammatory mediators include cytokines, vasoactive amines, proteolytic enzymes, vasoactive peptides, chemokines, lipid mediators, and complement component fragments. The nature of the inflammatory pathway initiated depends on the type of stimulus initiating inflammation [171, 172]. Cytokines are the principal organizers of the inflammatory response, determining cellular activation, infiltration, and systemic response [173]. Lipid mediators, such as eicosanoids, are also essential in many physiological processes, including inflammation [174].

Network pharmacology is an evolving field that combines systematic medicine and information science to enhance drug discovery and development [175]. Using computational biological methods to connect genomic technology with system biology, network pharmacology aids in comprehending the intricate relationships between biological systems, medications, and illnesses [176]. Network pharmacology enables an objective examination of target spaces to identify new drug leads and targets; also, it allows one to understand how drugs function and interact with various targets [177]. Network pharmacology is a benchmark for screening bioactive compounds in medicinal plants and offers innovative alternatives to traditional medicine [178]. By combining advances in systems biology and network pharmacology, the next generation of

promising medications could be rationally created, addressing the complexity of NP metabolomes [175].

Protein-protein interactions (PPIs) are physical interactions between protein molecules in biological systems, crucial for cellular functions and regulation. Understanding PPIs is essential for understanding cellular pathways, signal transduction, and biological function regulation [179]. Knowing PPIs is crucial in network pharmacology, as they dictate protein behavior and biological processes through a complex interplay of short-range and long-range forces. Understanding these interactions provides insights into protein regulation and dynamics in cellular processes [180].

Natural lactones represent a promising class of compounds for developing new anti-inflammatory drugs. While there is growing scientific evidence to support the use of natural lactones as new therapeutic agents for treating inflammation and inflammatory diseases, detailed research is required to thoroughly know their mechanisms of action. The study aimed to identify lead anti-inflammatory lactones from the Sistemat X database of secondary metabolites using network pharmacology, gene enrichment, molecular docking, and *in vitro* approaches against inflammation.

6.2 Experimental section

6.2.1 Dataset curation

The dataset of lactones was obtained from Sistemat X database of secondary metabolites (https://sistematx.ufpb.br). In database, the properties filter was used, and sesquiterpene lactones with five-membered lactone rings were filtered out from the lactone database. This filtering resulted in 1800 molecules with a five-membered lactone ring. These molecules were subjected to further filtering using KNIME analytics platform to remove broken molecules and PAINS. PAINS molecules are expected to provide the false result by interacting with numerous biological targets in a nonspecific manner rather than a

desired single specific target. It was followed by Brenk filtering and Lipinski's rule of five violations. Brenk filter identifies metabolically unstable, chemically reactive substances with poor pharmacokinetic properties, ensuring adequate pharmacokinetic properties, metabolic stability, tolerable hazardous levels, and chemical inactivity. The RDKit molecule catalogue filter node was used for this purpose.

6.2.2 *In silico* assessment of pharmacokinetic parameters

As mentioned in Section 3.2.8.

6.2.3 Identification of disease target genes

The chosen compounds were subjected to target prediction using SwissTargetPrediction program (http://www.swisstargetprediction.ch) [181]. It's crucial to discover the genes linked to disease in order to build the compound-target network. The target genes linked with inflammation were identified using data from the human gene database Uniprot (https://www.uniprot.org/), which offers details on all annotated and predicted human genes. The probability of gene matching was set to >0.05, and only the genes active against chosen compounds were selected.

6.2.4 Compound target network construction

Following the completion of the PPI network, the compound-target network was built using Cytoscape visualization software version 3.7.1 in order to better understand the chemical mechanism [182]. The compound-target network aids in deciphering and analyzing the target's mode of action and pathway involved.

6.2.5 Target genes identification and PPI network building

Understanding the role that proteins play in diverse biochemical processes is vital for understanding cellular organization, bioprocesses, and functions. The understanding of the complex web of PPIs between inflammatory gene proteins is crucial for deciphering the intricate mechanisms underlying inflammatory processes [183]. In PPI, short and

long-range interactions play a crucial role. Short-range interactions in PPIs involve direct physical contact between amino acid residues, contributing to molecular recognition, stability, and functionality of protein complexes [184]. These interactions include hydrogen bonding, ionic interactions, van der Waals forces, hydrophobic interactions, pi-stacking, and cation-π interactions. Long-range interactions in proteins involve amino acid residues distant from the protein's structure, influencing its dynamics and flexibility [180]. These interactions stabilize or destabilize specific structural elements, allowing proteins to undergo conformational changes. They are crucial for functional switching in multi-domain proteins and transmitting information over extended distances [185]. The STRING 12.0 virtual screening database (https://string-db.org/) was used for this purpose [186]. The genes of the selected lactones were submitted to STRING to build and learn about PPI. The network was generated for "*Homo sapiens*," and the confidence level of target protein interaction was set to >0.9 (highest confidence data). Proteins were represented by nodes of the network, and related PPIs were represented by the edges. Further, the PPI network was imported into Cytoscape and here only those genes were selected that had degree of interaction 2 or above. This produced a total of 10 final genes.

6.2.6 Analysis of gene ontology (GO) gene richment and Kyoto encyclopedia of genes and genomes (KEGG) pathway

ClueGO is an additional Cytoscape plug-in that performs GO and KEGG pathway annotation and offers a network-based visualization to minimize duplication of results from pathway enrichment analysis [187]. GO is used to analyze the gene cluster in the network to enhance data prediction. GO provides a hierarchically organized set of thousands of standardized terms for biological processes, molecular activities and cellular compounds, with verified and anticipated gene annotations based on these words for many species. A common resource for route enrichment analysis is GO annotation. The

goal of the investigation is to pinpoint the biological mechanism that will allow for the layout of useful functional data. KEGG is used to determine the metabolic route and gene functions of the inputted gene and molecule network. It can also be used to identify the disease-related target's contributory route. The final genes found were imported to CleuGo extension of Cytoscape for GO and KEGG pathway analysis.

6.2.7 Docking of the active compounds

Using the AutoDock 4.0 tool, docking study was conducted to determine the binding mode and affinity of the chosen active compound. The chosen proteins included PTGES, PTGS1, PTGS2, ALOX5, and IL1B. The COX-1 and COX-2 proteins were also chosen for docking as PTGS1 and PTGS2 encode for COX-1 and COX-2 proteins, respectively. The RCSB Protein Data Bank was used to obtain the crystal structures for COX-2 (PDB Id: 3NT1), COX-1 (PDB Id: 3N8X), 5-LOX (PDB Id: 6NCF), Interleukin-1-beta (PDB Id: 9ILB), prostaglandin E synthase 1 (PDB Id: 3DWW), prostaglandin E synthase 2 (PDB Id: 1Z9H), which were then processed for docking using AutoDock.

6.2.7.1 Docking studies

As mentioned in Section 4.2.3.2.

6.2.8 MD simulation study

Using the Desmond package (D.E. Shaw Research, New York, USA) with an integrated optimal potential for liquid simulations (OPLS 2005) force field, the MD simulation of STX 12273-COX-2 complex was performed. The STX 12273-COX-2 complex was obtained from AutoDock post docking. The solvated system was built using an open TIP3P water model with periodic boundary conditions in a cubic box with 12 X 12 X 12 Å dimensions prior to the MD simulation. The overall negative charge of the solvated system was neutralized by adding sodium ions. The desired electrically neutral system for simulation was created using 0.15 M NaCl. The steepest descent method and the

Broyden-Fletcher-Goldfarb-Shanno algorithm with a threshold of 2.0 kcal/mol were used to relax the system. The simulation, using Berendsen thermostat and barostat approaches, was run for 120 ns in an NPT ensemble. Throughout simulation run, the Noose-Hoover thermostat algorithm and the Martyna-Tobias-Klein Barostat algorithm were used to maintain a constant temperature of 310 K and a constant pressure of 1 atm, respectively. The short-range coulombic interactions were analyzed using the short-range method by setting the cut-off value of 9.0 Å. The long-range coulombic interactions were handled using the smooth particle mesh Ewald method and the tolerance value was set to 10^{-9} which was implemented by the SHAKE algorithm. The final production run was carried out for 120 ns and the trajectory sampling was done at 100 ps. The trajectories obtained from the following MD run were analyzed for protein and ligand RMSD, RMSF, and PL-contacts.

6.2.9 *In vitro* experimental validation using COX-2 enzymatic assay

Following MD simulation, the hit was evaluated for *in vitro* COX-2 inhibition. The COX (ovine) Inhibitor Screening Assay kit was used to measure the COX-2 inhibition activity (Catalogue No. 760111; Cayman Chemical, Michigan, USA). The assay was performed in accordance with the instructions supplied by the manufacturer [188]. By observing the emergence of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), the assay calculates the heme-catalyzed hydroperoxidase activity of ovine COX-2. The resulting concentration-inhibition (%) plots, fitted to sigmoid curves, were used to determine the IC_{50} value [189].

6.3 Result & Discussion

6.3.1 Lactone compounds in Sistemat X database

The Sistemat X NP database comprised 1800 molecules with a five-membered lactone ring. After rigorous filtering (PAINS, Brenk, Lipinski's Rule of Five) 242 molecules were

obtained. Later, the *in silico* ADMET profiling of these molecules was carried out and after removing the molecules showing Ghose, Veber, Muegge, Egan violations, and Cytochrome P450 violations, the final 30 molecules were remaining. The target prediction of these 30 molecules via SwissTargetPrediction showed that only 27 compounds were active. The Uniprot human gene database was used to retrieve the genes associated with inflammation, whereas the genes of these 27 compounds were obtained by Swiss target prediction. Only the genes with known activity were selected (with probability > 0.05), resulting in 38 unique targets. Only those compounds that showed activity against inflammatory targets were chosen for further analysis which resulted in 24 compounds.

6.3.2 Compound-target network

The data was loaded into Cytoscape to create a compound-target network, which allowed evaluation of the signalling route and target gene activity. The compounds and gene targets interaction network created in Figure 6.1 clarifies the mechanisms of therapeutic action of these compounds. It has 257 interacting target proteins, 24 compounds, and several compounds showed interactions with many targets. This finding suggested that the active natural lactones may have a synergistic effect on these targets; in addition to treating inflammation, it also possesses therapeutic benefits on other diseases and disorders.

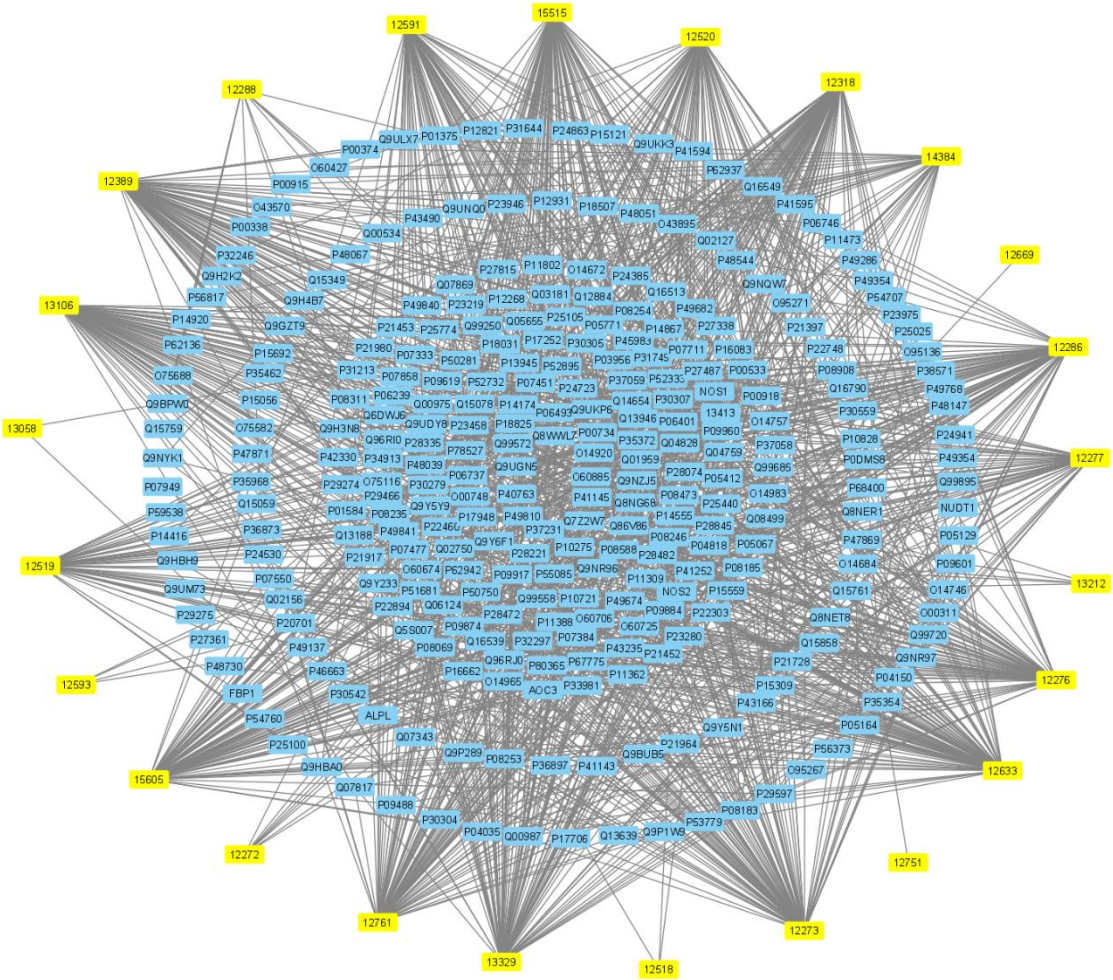

Figure 6.1 Compound-target network build using Cytoscape v_3.9.1.

6.3.3 PPI network

Studying the PPI of the target genes is crucial for improving visualization and comprehending the targets' mechanisms. To visualize and build the PPI network for the related drugs, the target genes were put through STRING v 12.0. A score level more than 0.9 was used to set the high-confidence target protein interaction data. The interactions between the target proteins are depicted in Figure 6.2. There are 75 edges and 23 nodes; each edge represents a PPI.

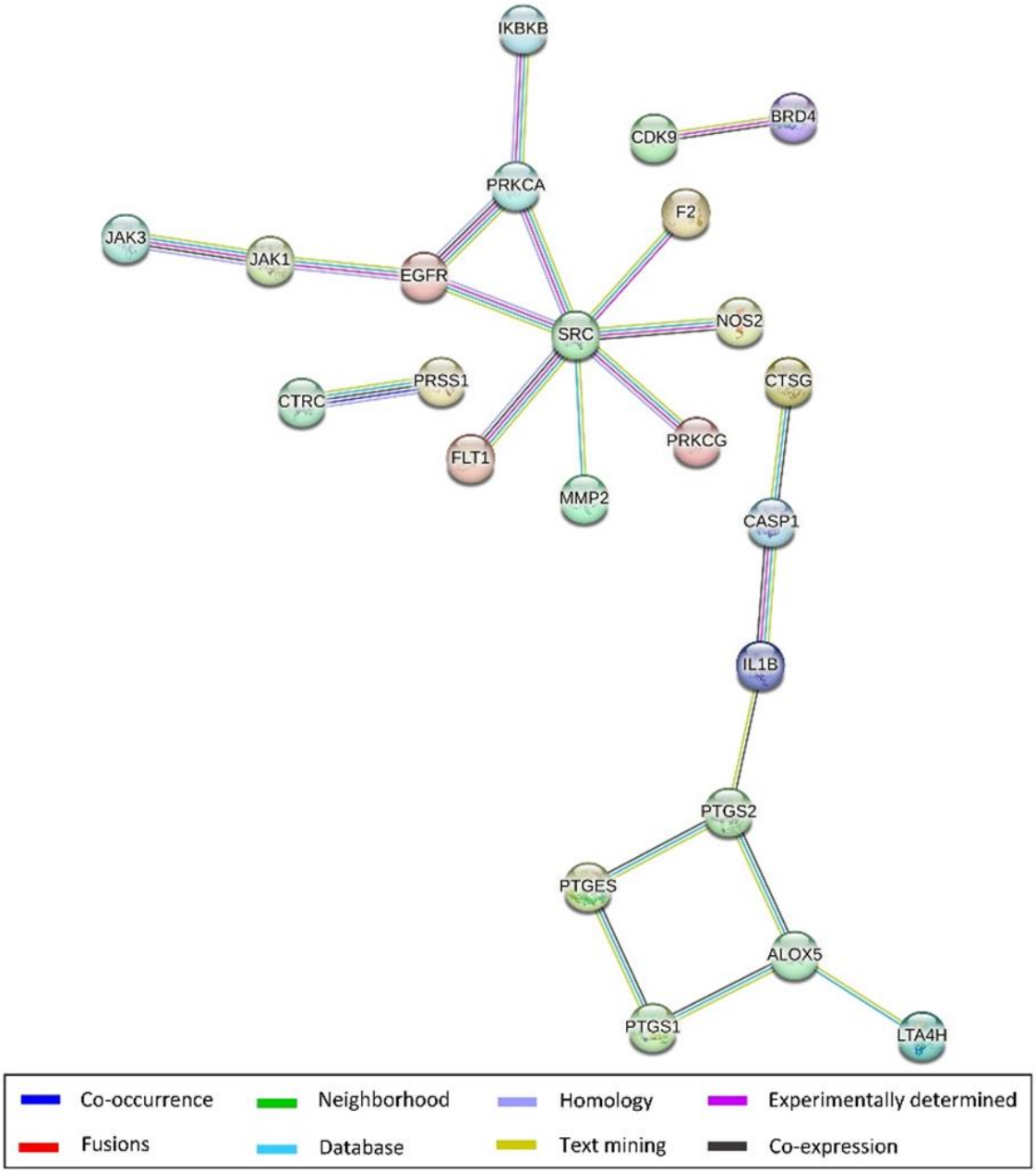

Figure 6.2 PPI network of inflammatory targets build using STRING v. 12.0 (Color represents type of PPI, nodes represent protein, and edges represent interaction).

PTGS2, IL1B, PRKCA, PTGES, SRC, PTGES1, CASP1, ALOX5, and EGFR were identified as the major targets involved in inflammation, through PPI network. Among them, PTGES, PTGS1, PTGS2, SRC, IL1B, and ALOX5 were located centrally indicating these proteins are crucially involved in pathogenesis of inflammation (Figure 6.2). Primarily, the PTGES, ALOX5, IL1B, PTGS1, and PTGS2 pathways are key starting points for determining the mechanisms of inflammation. They encode various

proteins and enzymes that are involved either in the initiation, development, or progression of inflammation. So, the PPI network and pathway analysis of inflammatory genes was done to recognize critical genes related to inflammation. Further, the PPI network was analyzed for critical information as mentioned in Table 6.1.

Table 6.1 Significant nodes with findings from network analyzer.

Name	Betweenness Centrality	Closeness Centrality	Clustering Coefficient	Degree	Topological Coefficient	Radiality
PRKCG	0.0	0.434782	0.0	1	0.0	0.814286
BRD4	0.0	1.0	0.0	1	0.0	1.0
JAK3	0.0	0.303030	0.0	1	0.0	0.671428
PRSS1	0.0	1.0	0.0	1	0.0	1.0
CTSG	0.0	0.291666	0.0	1	0.0	0.190476
PRKCA	0.2	0.555556	0.333333	3	0.416667	0.885714
LTA4H	0.0	0.35	0.0	1	0.0	0.380952
NOS2	0.0	0.434783	0.0	1	0.0	0.814286
IKBKB	0.0	0.370370	0.0	1	0.0	0.757143
PTGES	0.095238	0.4375	0.0	2	0.75	0.571428
CASP1	0.285714	0.388889	0.0	2	0.5	0.476190
JAK1	0.2	0.416667	0.0	2	0.5	0.799999
FLT1	0.0	0.434783	0.0	1	0.0	0.814286
ALOX5	0.380952	0.5	0.0	3	0.5	0.666667
PTGS1	0.047619	0.388889	0.0	2	0.75	0.476190
CDK9	0.0	1.0	0.0	1	0.0	1.0
SRC	0.777777	0.714286	0.047619	7	0.214286	0.942857
EGFR	0.355555	0.588235	0.333333	3	0.407407	0.9
IL1B	0.476190	0.5	0.0	2	0.5	0.666667
PTGS2	0.619047	0.583333	0.0	3	0.444444	0.761905
MMP2	0.0	0.434783	0.0	1	0.0	0.814286
CTRC	0.0	1.0	0.0	1	0.0	1.0
F2	0.0	0.434783	0.0	1	0.0	0.814286

6.3.4 Interactions between proteins from PPI network

Major target proteins in inflammation, identified through the PPI network, exhibit potential short (direct) and long-range (indirect) interactions. The plausible interactions are mentioned as follows: PTGS2 directly interacts with PTGES to convert PGH2 into PGE2, a key inflammatory mediator [190]. PTGS2 also binds to AP-1 transcription factor complex, enhancing its pro-inflammatory gene expression [191]. PTGS2 indirectly interacts with IL1B and TNF-α through downstream PGE2 signaling, amplifying the inflammatory response [192]. PTGS2 can be regulated by phosphorylation through kinases like SRC and PRKCA, affecting its activity and stability [193]. IL1B via short-range interactions, binds to IL1R1 on target cells, triggering downstream inflammatory signaling cascades [194]. IL1B via short-range interactions, induces PTGS2 expression, leading to increased PGE2 production and amplifying inflammation [192]. It triggers the release of pro-inflammatory cytokines like IL6 and TNF-α, leading to a cascade of inflammatory responses [195]. PRKCA directly phosphorylates and activates various proteins involved in inflammatory signaling, including MAPKs and NF-κB. It interacts with and regulates the activity of other kinases like SRC and EGFR [196, 197]. It indirectly contributes to the upregulation of genes like PTGS2 and IL1B, promoting sustained inflammation.

PTGES is closely associated with PTGS2 in the endoplasmic reticulum, enabling efficient conversion of PGH2 to PGE2. PTGES is indirectly involved in determining the local PGE2 levels that influence various inflammatory processes (vasodilation, edema, pain). PTGES also contributes to the development and progression of chronic inflammatory diseases [190]. SRC directly phosphorylates and activates signaling proteins, including PTGS2, EGFR, and MAPKs, amplifying inflammatory responses. It indirectly plays a crucial role in cell migration and adhesion, critical for leukocyte infiltration during

inflammation. It is also involved in cell proliferation and survival, potentially contributing to inflammatory tissue damage [198, 199]. PTGES1 is primarily localized in the cytosol and contributes to the PGE2 synthesis. It is potentially involved in regulating cell apoptosis and inflammatory resolution via long-range interactions [190]. CASP1 plays a central role in the inflammasome, a protein complex responsible for processing and activating inflammatory cytokines like IL1B and IL18. It interacts with adaptor proteins and pro-inflammatory cytokine precursors within the inflammasome. CASP1 is indirectly and critically involved in pyroptosis, a programmed cell death mechanism associated with certain inflammatory responses. It also influences the balance between pro-inflammatory and anti-inflammatory signals within the immune system [200]. ALOX5 converts arachidonic acid into leukotrienes, potent inflammatory lipid mediators. It interacts with and is regulated by various regulatory proteins and cofactors [201].

6.3.5 Gene neighbouring effect of identified genes

The gene neighboring or cis-regulatory effect refers to the influence of nearby genes on the target gene expression [202]. This effect could be mediated through various mechanisms like promoter sharing, enhancer-promoter interactions, and chromatin modifications [203-205]. In PPI network, understanding gene neighboring effects can provide valuable insights into the regulation of gene expression within functional modules [206]. The possible neighboring genes influencing the expression of the identified anti-inflammatory genes through the PPI network are mentioned below.

PTGS2 expression has been reported to be regulated by neighboring genes like PLA2R1 and TBX2 through promoter sharing and enhancer-promoter interactions [207, 208]. IL1B expression could be influenced through chromatin modifications and enhancer-promoter interactions via neighboring genes like NLRP3 and TNF-α [209, 210]. The gene expression of PRKCA could be regulated by neighboring genes PRKCBP1 and MAPK8

through promoter sharing and enhancer-promoter interactions [211]. Neighboring genes PTGS2 and COX1 can influence PTGES expression through promoter sharing and enhancer-promoter interactions [212]. In the case of SRC, neighboring genes like FYN and YES1 can influence SRC expression through promoter sharing and enhancer-promoter interactions [213]. PTGES1 expression could be influenced by neighboring genes PTGS2 and COX1 via promoter sharing and enhancer-promoter interactions [212]. Neighboring genes like NLRP3 and ASC can influence CASP1 expression through promoter sharing and enhancer-promoter interactions [214]. ALOX5 expression could be influenced by neighboring genes like ALOX12 and ALOX15 through promoter-sharing and enhancer-promoter interactions [215]. Neighboring genes like ERBB2 and PIK3CA can influence EGFR expression through promoter sharing and enhancer-promoter interactions [216].

6.3.6 Analysis of GO gene enrichment and annotation of KEGG pathways

The target proteins were examined using GO enrichment analysis. Three criteria were used for the ClueGO functional analysis in order to assess the target genes for the KEGG pathway, GO molecular function, and GO biological process (Table 6.2 and). Network specificity was set to medium, and the GO term fusion was limited to p-value ≤0.005, which is based on the false discovery rate (Benjamini-Hochberg). To identify the signaling mechanisms of inflammation, GO and KEGG analysis was performed and following mechanisms were found to be connected with inflammation- regulation of fever generation, heat generation, prostaglandin biosynthetic process, oxidoreductase activity, icosanoid biosynthetic process (Figure 6.3). Out of 10 genes used for GO enrichment analysis, only 8 showed interaction with related inflammatory mechanisms (Figure 6.4). Among 8 genes, only those genes were selected that showed interaction with more than five inflammatory pathways. This resulted in the final 5 genes (IL1B, ALOX5, PTGES1,

PTGES2, and PTGES) and 18 lactone compounds from Sistemat-X that showed interactions with these targets.

Table 6.2 GO biological process.

Description	**Count in gene set**	**False Discovery Rate**
Primary metabolic process	23	3.30×10^{-7}
Organic substance metabolic process	23	8.31×10^{-7}
Cellular metabolic process	22	6.83×10^{-6}
Response to stimulus	21	1.90×10^{-4}
Organonitrogen compound metabolic process	20	2.72×10^{-6}
Macromolecule metabolic process	20	2.57×10^{-5}
Cellular response to stimulus	20	5.07×10^{-5}
Response to stress	19	1.87×10^{-7}
Protein metabolic process	19	1.07×10^{-6}
Response to chemical	18	1.07×10^{-5}
Positive regulation of biological process	18	7.00×10^{-4}
Response to organic substance	17	8.31×10^{-7}
Regulation of response to stimulus	17	3.21×10^{-5}
Positive regulation of cellular process	17	9.50×10^{-4}
Cellular protein metabolic process	16	5.41×10^{-5}
Signal transduction	16	9.50×10^{-4}
Multicellular organismal process	16	3.27×10^{-2}
Response to external stimulus	15	2.58×10^{-6}
Cellular response to chemical stimulus	15	2.62×10^{-5}
Regulation of cell communication	15	1.90×10^{-4}
Regulation of signaling	15	2.10×10^{-4}
Negative regulation of biological process	15	9.50×10^{-3}
Regulation of cellular metabolic process	15	3.68×10^{-2}
Regulation of macromolecule metabolic process	15	4.64×10^{-2}
Cyclooxygenase pathway	3	1.30×10^{-4}

Table 6.3 GO molecular function.

Description	Count in gene set	False Discovery Rate
Catalytic activity	22	1.99×10^{-8}
Binding	22	3.91×10^{-2}
Ion Binding	19	2.80×10^{-4}
Catalytic activity, acting on a protein	17	6.88×10^{-9}
Anion binding	13	7.60×10^{-4}
Carbohydrate derivative binding	12	5.90×10^{-4}
Protein kinase activity	10	4.04×10^{-7}
Ribonucleotide binding	10	4.00×10^{-3}
Enzyme binding	10	1.17×10^{-2}

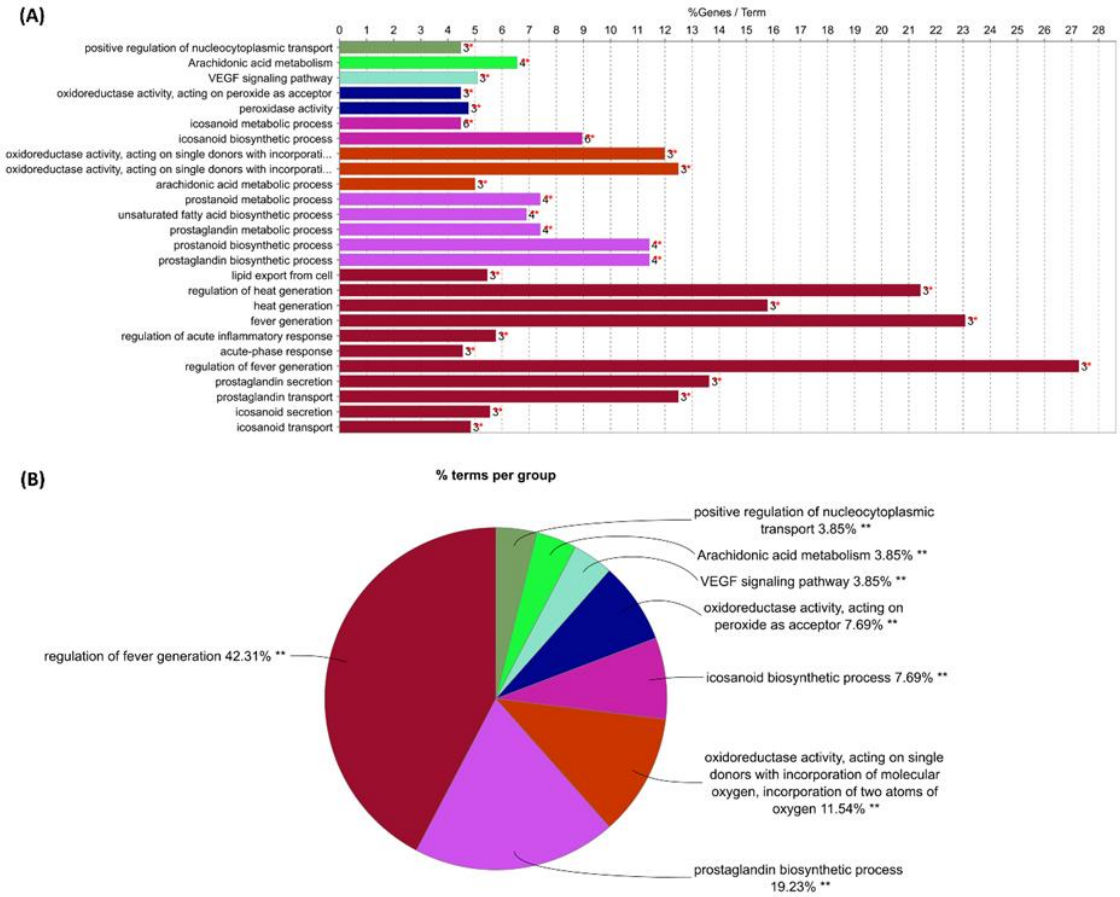

Figure 6.3 Gene enrichment analysis. (A) Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis of inflammatory proteins and (B) Gene ontology (GO) enrichment analysis of inflammatory proteins.

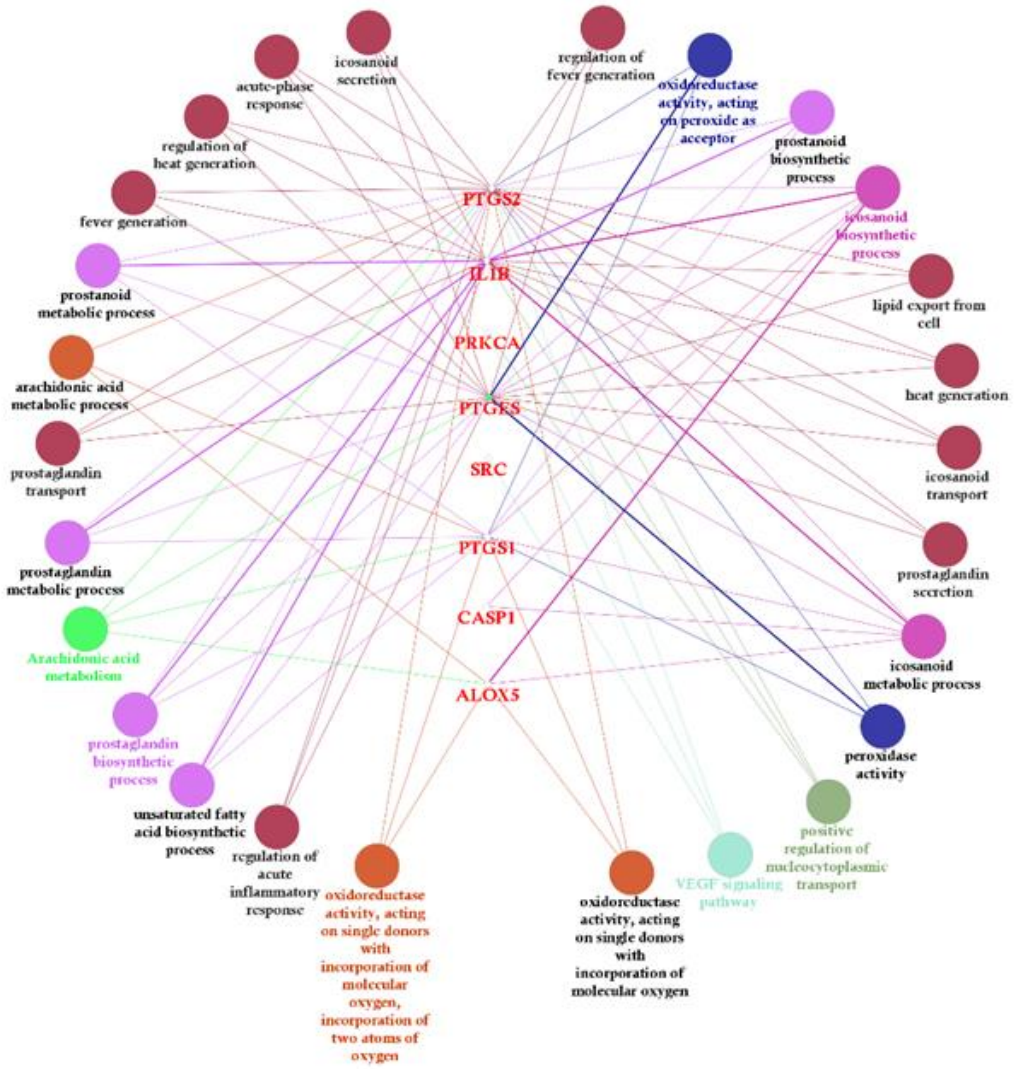

Figure 6.4 Major genes of inflammation and their related inflammatory pathways.

6.3.7 Molecular docking of the active lactones

Molecular docking of the identified lactones was carried out after the pathways and illnesses associated with genes were analyzed, to predict their binding conformation with the target binding site. The proteins were chosen on the basis of their crucial involvement in PPI, compound-target network, and GO analysis, and inflammation. The docking score of 18 compounds with five crucial inflammatory proteins- COX-2 (PDB Id: 3NT1), COX-1 (PDB Id: 3N8X), 5-LOX (PDB Id: 6NCF), IL-1β (PDB Id: 9ILB), prostaglandin E synthase 1 (PDB Id: 3DWW), prostaglandin E synthase 2 (PDB Id: 1Z9H)) has been shown as a heatmap in Figure 6.5. Amongst all the compounds and proteins, STX 12273

showed best binding energy with COX-2 protein particularly. The interaction diagram of the best compound STX 12273 with COX-2 is shown in Figure 6.6.

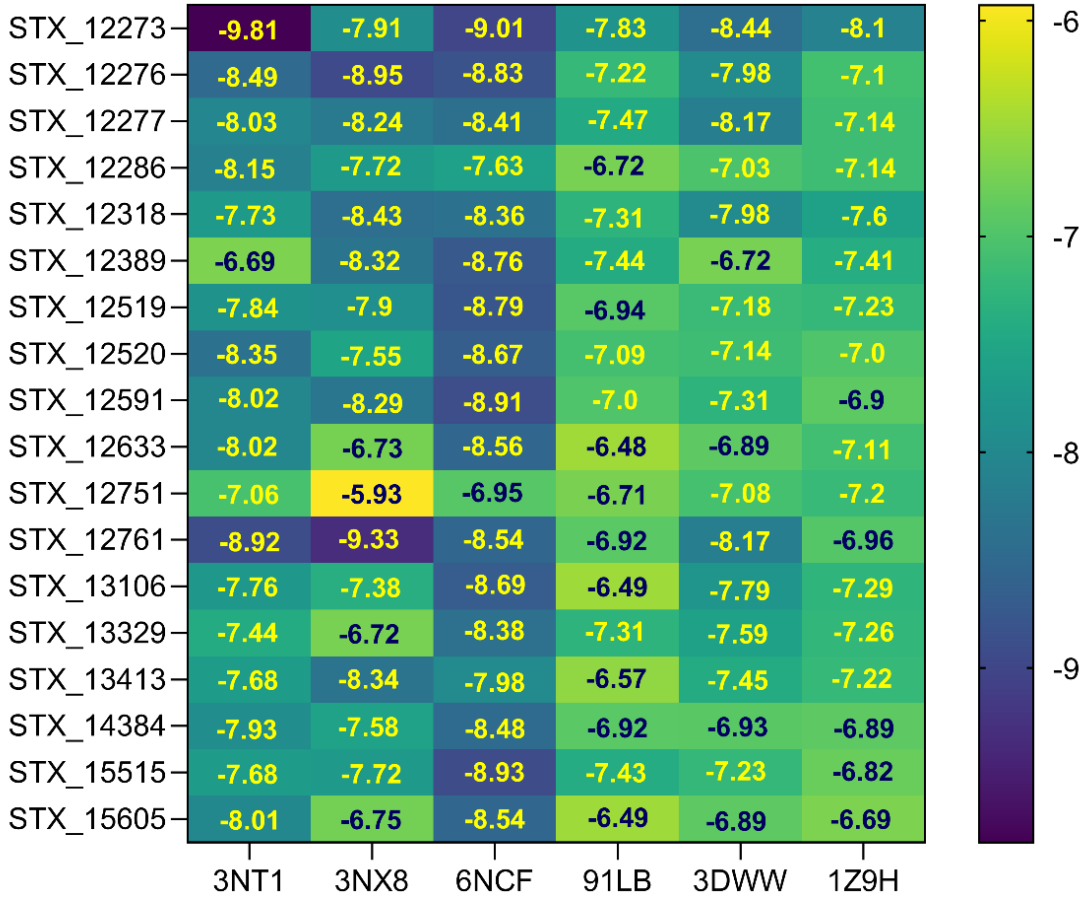

Figure 6.5 Heatmap of docking score of active lactones against inflammatory targets.

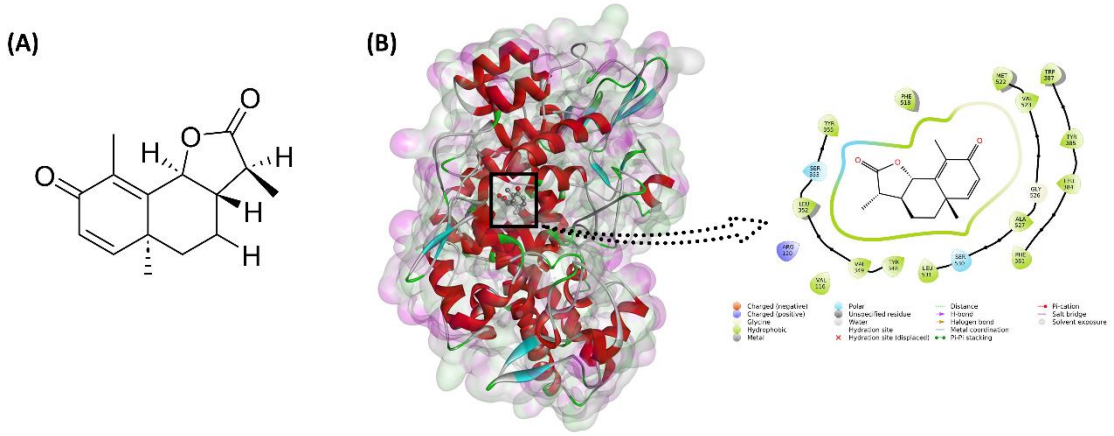

Figure 6.6 Docking study of STX 12273 with COX-2 protein (PDB: 3NT1). (A) Structure of STX 12273 and (B) 2D interaction diagram.

6.3.8 MD simulation studies

As STX 12273 showed the best binding energy with COX-2 among all the compounds and proteins, the MD simulation study of STX 12273 was conducted with COX-2 to analyze the stability of the complex in a simulated environment. The simulation trajectories were analyzed for RMSD, RMSF, PL-contacts, and ligand properties.

The RMSD value of the C-alpha backbone of COX-2 and STX 12273 was determined during a simulation run for 120 nanoseconds. The protein showed stability throughout the run with an average C-alpha backbone RMSD of 0.57 Å. The ligand also displayed stability during the run with an average RMSD of 0.72 Å (Figure 6.7). As the ligand RMSD was closer to that of protein RMSD, representing that the ligand did not diffuse away from the active site of the protein.

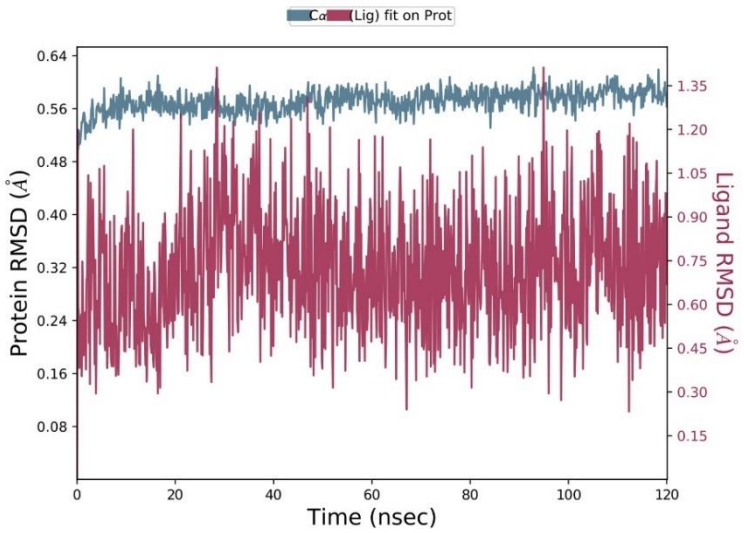

Figure 6.7 RMSD plot of STX 12273- COX-2 complex.

The P-RMSF and L-RMSF help in characterizing the local changes occurring in the protein backbone and ligand atoms. The P-RMSF and L-RMSF analysis was conducted for ligand fit on protein and C-alpha atoms of protein residues, respectively. The average P-RMSF (Figure 6.8) and L-RMSF (Figure 6.8) for STX 12273-COX-2 were 0.36 Å and 0.48 Å, respectively.

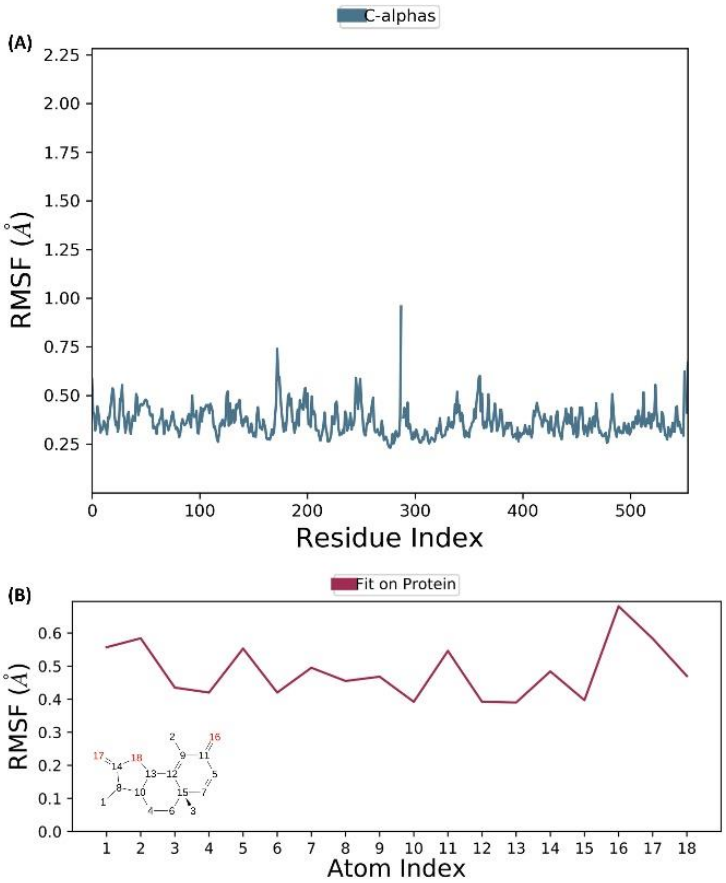

Figure 6.8 RMSF plot of STX 12273 complexed with COX-2. A) P-RMSF plot representing residue-wise deviation in STX 12273- COX-2 complex and B) L-RMSF plot representing atom-wise deviation in STX 12273 bound to COX-2 protein.

The interactions between COX-2 protein and STX 12273 during the simulation were analyzed by PL-Contacts histogram (Figure 6.9A). The ligand displayed various interactions with protein residues like hydrophobic, ionic, water bridges, and hydrogen bonds. STX 12273 showed various interactions with Val 349, Leu 352, Trp 387, and Phe 518 which were seen in the docking interaction also. The timeline display of interactions and PL-Contacts is summarized in Figure 6.9B. The overall specific interactions between protein and ligand are shown in the upper panel, while the residue interaction with ligand in each frame is shown in the lower panel. The image demonstrates the pattern of residue contacts like Leu 352 and Trp 387 with STX 12273. The darker shade of orange indicates that residues are interacting with ligand through more than one contact. Figure 6.9C

indicates the residues that interacted most with the specific ligand group during the simulation.

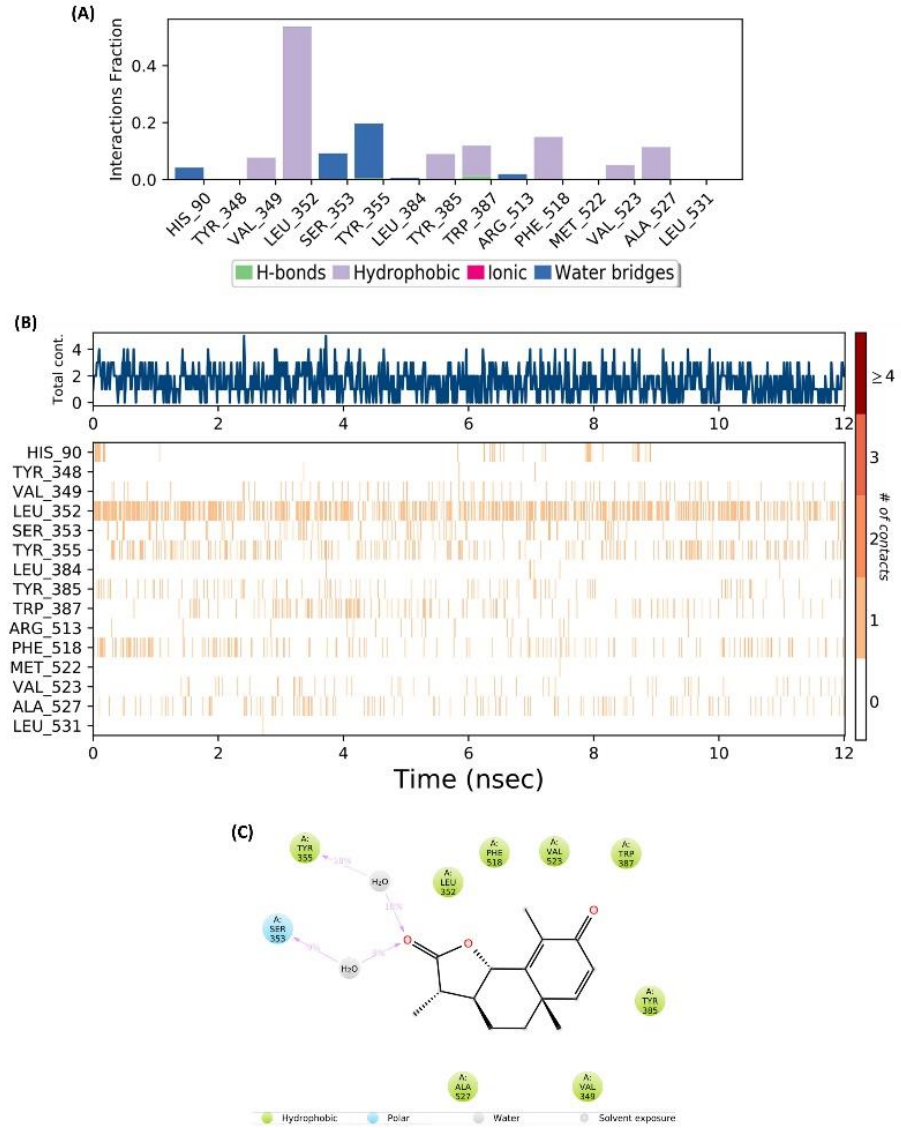

Figure 6.9 Protein-ligand contacts of STX 12273 with COX-2 protein during MD simulation. A) The STX 12273- COX-2 complex's PL-Contacts during simulation, B) A timeline depiction of the interaction between the COX-2 and STX 12273 complex, and C) The interaction between STX 12273 and the COX-2 protein.

Figure 6.10 displays the ligand properties of STX 12273, which include RMSD, radius of gyration (rGyr), solvent accessible surface area (SASA), molecular surface area (MolSA), and polar surface area (PSA). At time *t=0*, the divergence of ligand from its reference conformation is shown by ligand RMSD. It was observed that STX 12273

showed only small deviation with minimum, maximum, and average deviation of 0.093 Å, 0.296 Å, and 0.173 Å, respectively indicating its stability throughout the MD run. The ligand extendedness was measured using the radius of gyration (rGyr) to determine the ligand stability during simulation. The rGyr trajectory of STX 12273 showed a small deviation between 2.87 Å and 3.016 Å indicating that the ligand remained stable during MD. No intraHB formation in the ligand was observed during simulation. MolSA is similar to van der Waals surface area and the average MolSA for STX 12273 was found to be 236.269 $Å^2$. The surface area of ligand accessible to water molecule of simulation system is measured by SASA and STX 12273 showed average SASA value of 7.009 $Å^2$ ranging between 0.486 $Å^2$ to 22.588 $Å^2$. The solvent-accessible surface area of a ligand donated only by nitrogen and oxygen atoms is represented by PSA. The average PSA value for STX 12273 was found to be 86.971 $Å^2$ ranged from 81.761 $Å^2$ to 92.959 $Å^2$.

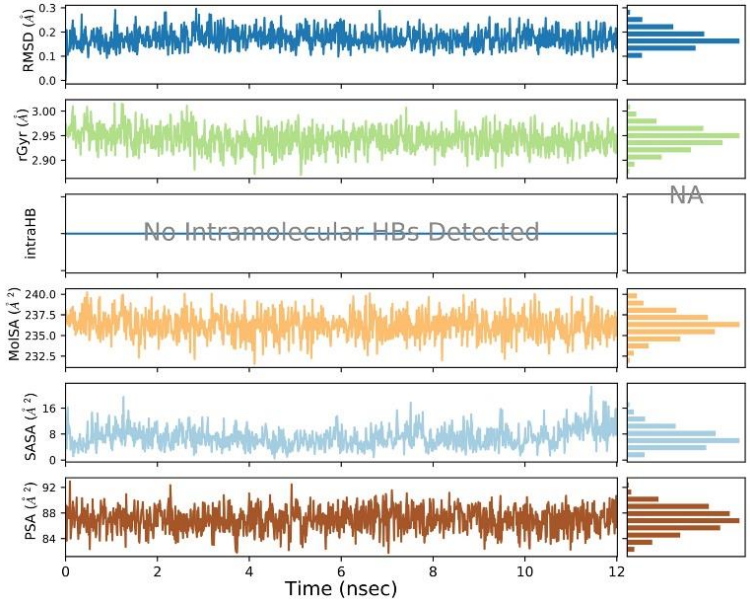

Figure 6.10 Ligand properties of STX 12273 complexed with COX-2 during the MD run.

Increasing SASA generally correlates with higher RMSF, as more solvent exposure often implies greater flexibility and movement [217]. The relationship between SASA and RMSF in the context of PPI is complex as both provide valuable insights into protein behavior and potential interaction mechanisms. PPI interfaces typically involve areas

with complementary shapes and functional groups, often characterized by changes in SASA relative to the unbound state. In PPI interfaces, areas with high RMSF may facilitate conformational adjustments for optimal interaction and complex formation [218, 219]. SASA and RMSF together can provide more comprehensive insights into potential PPI interfaces. Areas with high SASA and RMSF could be hotspots for interaction due to their solvent exposure and flexibility [220]. While a general correlation exists between SASA and RMSF, their relationship in PPI is more complex and requires careful interpretation considering the structural and functional aspects.

The relationship between SASA and gene neighboring effect in PPI is complex and multifaceted. During PPI, changes in SASA can be influenced by gene neighboring effects. Gene neighboring-mediated changes in SASA, particularly in critical interfacial regions, can affect the protein's ability to bind or interact with specific partners, impacting its overall function within the network [220]. Changes in the conformation or activity of a protein due to neighboring gene influence can modify its SASA, leading to altered binding affinities with other proteins. The impact of gene neighboring effect on SASA and PPI can be tissue-specific and vary according to developmental stage and environmental factors. Analyzing SASA changes alongside gene neighboring effects can provide insights into potential PPI partners and functional modulation [218].

6.3.9 *In vitro* experimental validation of COX-2 inhibition by STX 12273

The PPI network, compound-target network, and GO enrichment analysis revealed the crucial role of COX-2 (PTGS2) in inflammation and its mechanisms. Among the 18 final lactone compounds, STX 12273 showed the best results against COX-2 as revealed by detailed molecular docking and molecular modeling studies. The structure of hit STX 12273 corresponded to santonin, a sesquiterpene lactone. Hence, the *in silico* results were

validated by evaluating the inhibition of COX-2 by STX 12273 in an *in vitro* enzymatic assay. The IC_{50} of STX 12273 and Celecoxib are provided in Table 6.4.

Table 6.4 COX-2 inhibitory activity of STX 12273.

Compound	**COX-2 inhibition (IC_{50} in nM) (Mean ± S.D.)**
STX 12273	622.20 ± 8.22
Celecoxib	416.33 ± 2.70

6.4 Conclusion

Network pharmacology is an attractive approach for investigating preliminary mechanisms and interactions of phytoconstituents with varied disease targets and aids in reducing time and cost of experimental screening. Network pharmacology-based analysis of the natural lactone database in this study revealed 18 hit lactones connected to inflammatory targets. PTGES, PTGS1 (COX-1), PTGS2 (COX-2), ALOX5 (LOX), and IL1B (IL-1β) were primary targets for inflammation. A total of 26 pathways involved in inflammation were identified. GO molecular function showed that highest number of genes, almost 39, are involved in catalytic activity and catalytic activity acting on protein. The inflammatory proteins analyzed by the compound-target network and PPI network were particularly enzymes with catalytic activity. The docking studies of 18 hits revealed STX 12273 as the best hit with lowest binding energy with COX-2. Further molecular modeling studies also revealed the stability of STX 12273-COX-2 complex in simulated biological environment. The potential of STX 12273 to inhibit the COX-2 enzyme was further confirmed by *in vitro* experimental validation via COX-2 inhibition assay. STX 12273 showed good inhibition potential against COX-2 *in vitro* with an IC_{50} value of 622 nM. This inhibition was also supported by a favorable binding energy of -9.81 kcal/mol obtained from docking studies of STX12273 against COX-2 protein. This study offers a detailed study of the five-membered lactone's putative mechanism of action in inflammation and suggests the potential of STX 12273 in the treatment of inflammation.