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EXPLORING ANTI INFLAMMATORY PROPERTIES OF *PHYLLANTHUS NIRURI*: NETWORK PHARMOCOLOGY ANALYSIS, INFLAMMATORY MODULATION INSIGHTS

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ABSTRACT

This comprehensive study utilizes an integrated approach, merging network pharmacology, molecular docking, and systems biology analyses, to uncover the anti-inflammatory potential of compounds derived from *Phyllanthus niruri*. Targets associated with *Phyllanthus niruri* were identified through various databases, and subsequent filtering using ADME analysis ensured a focus on targets with favorable drug likeness and oral bioavailability. Predicted targets for the filtered compounds were identified using Swiss Target Prediction and the Similarity Ensemble Approach (SEA).

Systematic target identification for various inflammatory diseases was conducted, leading to the construction of a detailed network in Cytoscape that unveiled common targets between inflammation and *Phyllanthus niruri*. The network analysis facilitated the identification of key compounds and key targets, guiding subsequent molecular docking studies. Our investigation revealed two promising compound-target interactions. Two compound-target complexes, determined by the highest docking scores, were selected for further exploration through molecular dynamics simulations. The investigation revealed intricate dynamics within these complexes, shedding light on their dynamic behavior and stability. Gene ontology analysis unveiled associations with oxidative stress, cell proliferation, growth, and signaling. Pathway analysis emphasized a significant correlation between inflammation and cancer, reinforcing the intricate interplay. Additionally, pathways related toinflammation such as TNF signaling pathway, IL-17 signaling pathway, Lipid and atherosclerosis were identified.

This research contributes valuable insights into the multifaceted effects of *Phyllanthus niruri* compounds on inflammation, presenting potential therapeutic avenues. The findings underscore the nuanced relationship within inflammation and various diseases, particularly cancer. The significance lies in the potential translation of these findings into future experimental validations and therapeutic interventions in the field of pharmacology.

Keywords: *Phyllanthus niruri*, Network pharmacology, Molecular docking, Inflammatory targets, Gene Ontology analysis, Cancer-associated pathways, Rheumatoid arthritis, Inflammatory bowel disease, ADME analysis, Drug likeness, Oral bioavailability

1. INTRODUCTION

An essential part of the immune system is inflammation, a sophisticated biological reaction meant to protect the body from damaging stimuli. Although inflammation serves a protective purpose, it can also be dysregulated and connected to the genesis of several illnesses, ranging from rheumatoid arthritis and inflammatory bowel disease to more dangerous and sometimes lethal diseases like cancer.

Comprehending the complex molecular processes that underlie inflammation and recognizing possible remedial measures are essential pursuits in modern pharmacological investigation.A vast array of medical issues are impacted by inflammation, which plays a major role in the pathophysiology of numerous diseases. Its significance extends far beyond the field of localized immune responses, as it becomes intricately linked to the development and progression of various disorders. Chronic inflammation has been recognized as a characteristic of cancer, providing an environment that is favorable to the development, growth, and spread of tumors. Although inflammation is a vital defensive mechanism against pathogens in infectious disorders, it can also cause tissue damage and worsen infections when it persists or becomes dysregulated. Furthermore, inflammatory processes are essential for the development of metabolic syndromes, autoimmune illnesses, neurodegenerative diseases, and cardiovascular problems. The wide range of disorders that inflammation is linked to highlights the importance of inflammation as a common denominator in various pathophysiological pathways. Therefore, it is critical to comprehend the complex link between inflammation and these different diseases in order to design tailored treatment strategies.

Numerous studies have demonstrated a clear correlation between the onset and progression of cancer and chronic inflammation. An environment that is conducive to cell division, genetic instability, and apoptosis evasion is created by prolonged inflammatory activity- all essential components of the genesis of cancer.Research indicates that pro-inflammatory mediators, such as cytokines, growth factors, and chemokines, are critical in promoting the development of cancer. Angiogenesis and tumor formation have been associated with significant inflammatory pathway actors such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).Moreover, nuclear factor-kappa B (NF-κB) activation, a critical regulator of inflammation that promotes cell survival and resistance to apoptosis, is often abnormally high in cancer cells. In addition to these genetic discoveries, epidemiological research has demonstrated the clinical significance of this association by demonstrating an increased risk of cancer in those with chronic inflammatory conditions.

Current treatments for inflammatory diseases primarily rely on the development ofNon-Steroidal Anti-Inflammatory Drugs (NSAIDs) like aspirin and ibuprofen, which, while

effective in alleviating symptoms, are accompanied by significant drawbacks. The chemical nature of NSAIDs and their synthetic availability raise concerns, particularly due to the prevalence of gastrointestinal side effects, cardiovascular risks, indigestion, and potential organ failures such as kidney failure. Despite being widely used, these synthetic medications are handled cautiously, highlighting the urgent need for alternate therapeutic approachesin the control of inflammation.

There is a growing awareness of the importance of natural substances and plant medicines in reaction to the drawbacks and possible side effects of conventional treatments. Present-day drugs, such as corticosteroids, immunosuppressive drugs, and NSAIDs,though providing symptomatic relief, exhibit limited efficacy and undesirable side effects, especially in the long term. Natural compounds derived from plants, historically employed in traditional medicine, present an appealing avenue for investigation due to their perceived therapeutic benefits and often lower incidence of side effects. The multifactorial nature of inflammatory diseases as well as the plethora of side effects associated with conventional medications demands a holistic approach, making the exploration of natural compounds, such as those from *Phyllanthus niruri*, particularly relevant. This calls for a paradigm shift towards embracing the potential of natural compounds as promising candidates for inclusion in the course of therapyfor inflammatory diseases, offering a safer and potentially more sustainable approach to managing these conditions.

For centuries, traditional medicine has valued *Phyllanthus niruri*, also referred to as the Chanca Piedra or "Stone Breaker," for its medicinal qualities. *Phyllanthus niruri*, a native of tropical areas, especially the Amazon rainforest, has a long history of of application in the management of certain ailments, from liver problems and digestive problems to kidney stones and UTIs. The plant's pharmacological effects are attributed to a wide range of bioactive substances, such as polyphenols, alkaloids, flavonoids, and lignans. The plant's amazing anti-inflammatory, antioxidant, antiviral, and hepatoprotective qualities have been revealed by recent scientific studies. Its ability to modify inflammatory pathways is particularly intriguing, since this makes it a viable option for therapeutic interventions in illnesses including cancer and inflammatory diseases that are characterized by dysregulated immune responses. As this project's integrated approach highlights, the chemicals produced from *Phyllanthus niruri* may interact with important inflammatory targets, providing insights into their potential in limiting immune escape and building a more favorable environment for immunological responses. *Phyllanthus niruri*'s diverse range of therapeutic applications highlights its significance in modern pharmacology and opens the door to more research and possible incorporation into medical procedures in the future.

While the anti-inflammatory and immunomodulatory properties of *Phyllanthus niruri* components have been discovered, yet little is known about their precise targets, routes, and modes of action. In our research project, we aim to bridge this knowledge gap by employing an integrated approach that explores the molecular dynamics, network interactions, and target proteins correlated with the anti-inflammatory effects of *Phyllanthus niruri* compounds. This

investigation seeks to unravel the intricacies of these properties, offering a strong basis for the creation of innovative therapeutic approaches in the field of inflammatory illnesses. *Phyllanthus niruri's* numerous medicinal uses highlight its importance in contemporary pharmacology and pave the pathway for additional research and possible incorporation into medical procedures.

To uncover the anti-inflammatory potential of chemicals produced from *Phyllanthus niruri*, this research employs a comprehensive and multidimensional strategy integrating network pharmacology analysis, in silico studies, gene ontology analysis, and pathway analysis. Network pharmacology helps uncover important participants in *Phyllanthus niruri*'s anti-inflammatory activities by clarifying the complex connections between chemicals and their target proteins inside biological networks. In order to guide future experimental validations, the in silico investigations, which make use of molecular docking and dynamics simulations, provide important insights into the binding interactions and dynamic behavior of these drugs with their target proteins. Geneontology study sheds light on the broader biological impacts of *Phyllanthus niruri* compounds on inflammatory processes by categorizing genes based on their activities. At the same time, pathway analysis reveals the interdependent biochemical pathways that these substances impact, offering a systems-level comprehension of their possible anti-inflammatory actions. When taken as a whole, these analyses provide a thorough picture of the molecular environment that *Phyllanthus niruri* compounds affect. They also offer important insights into the therapeutic potential of these compounds and serve as a roadmap for future pharmacological and experimental research on inflammatory diseases, particularly in relation to cancer and other related conditions.

The significance of this study lies in the urgent need to address existing gaps in our understanding of natural compounds' efficacy in managing inflammatory diseases. Given the complexity of chronic inflammation and the negative effects associated with non-steroidal anti-inflammatory medicines (NSAIDs), a more sophisticated therapeutic approach is required.The research scope extends to uncovering the intricate molecular interactions and pathways influenced by *Phyllanthus niruri* compounds, shedding light on their potential role in mitigating immune escape and offering novel avenues for intervention, particularly in the context of inflammation-associated diseases, including cancer. This project is pivotal in systematically identifying and characterizing the anti-inflammatory effects of *Phyllanthus niruri*, addressing the limitations of current treatments and providing a foundation for future experimental validations. The potential translation of these findings into therapeutic interventions holds promise for reshaping pharmacological approaches to inflammatory diseases and reinforcing the importance of natural compounds in contemporary medicine.

Our research project aims to uncover the compounds present in *Phyllanthus niruri* and their potential inflammatory targets. Thus, giving us insights on how does *Phyllanthus niruri* exerts its anti-inflammatory or inflammation modulatory effects on various inflammatory conditions such as cancer, rheumatoid arthritis, atherosclerosis etc.,

In this study, the objectives are delineated to systematically investigate the inflammation mitigatingpotential of compounds derived from *Phyllanthus niruri*. Firstly, identify and filter compounds through a rigorous process, employing various databases and ADME analysis to prioritize those with favorable drug likeness and oral bioavailability. Subsequently, find the targets present in inflammatory conditions and inflammation. Next, identify the targets that are relevant to inflammation by predicting the targets for the filtered compounds, obtaining targets with a high probability, andfind the targets that are relevant to inflammation, and constructing a network to unveil key targets and compounds. The investigation further delves into the molecular interactions by performing docking studies with identified key targets and compounds, prioritizing those with the highest scores. Additionally, molecular dynamics simulations are conducted to unravel the intricate dynamics and stability of the selected compound-target complexes. To provide a comprehensive understanding of the biological effects, gene ontology analysis is performed, shedding light on biological processes, cellular component and molecular functions. Furthermore, pathway analysis is undertaken to emphasize relationship between inflammation and other diseases and to identify pathways associated with other inflammatory conditions. These multifaceted objectives collectively contribute to bridging knowledge gaps, exploring the potential therapeutic avenues, and laying the groundwork for future experimental validations in the realm of pharmacology.

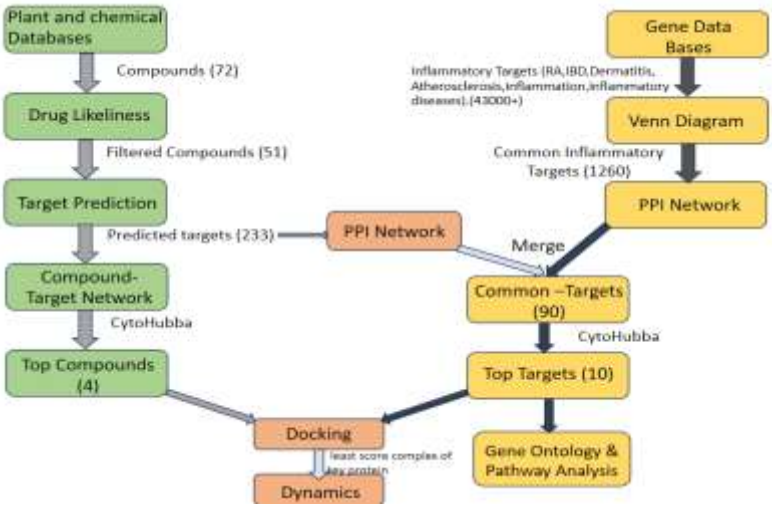

Figure 1: objective and workflow

.4.MATERIALS AND METHODS

4.1 Plant compounds collection and filtering based on DL and OB

Phyllanthus niruri's diverse range of bioactive chemicals was carefully collected using a thorough data mining approach that made use of reputable web sources and published literature on the phytochemistry of the plant (Bagalkotkar et al., 2006).This extensive dataset was prepared with use of databases such as the Dr. Dukes Phytochemical and Ethnobotanical Databases

(https://phytochem.nal.usda.gov/), Indian Medicinal Plants, Phytochemistry, and Therapeutics (IMPAAT-https://cb.imsc.res.in/imppat/home), ChEMBL (https://www.ebi.ac.uk/chembl/), Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (BATMAN-TCM-http://bionet.ncpsb.org.cn/batman-tcm/index.php), and KNApSAcK core system (http://www.knapsackfamily.com/knapsack_core/top.php).

Subsequently, the collected compounds underwent meticulous filtering based on drug likeness (DL) and oral bioavailability (OB). The Swiss ADME tool (http://www.swissadme.ch/) was used for this purpose. The canonical SMILES (Simplified Molecular Input Line Entry System) of the compounds were recognized from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and these were provided as input to the Swiss ADME tool.

The criteria for drug likeness were determined according to the Lipinski rule of five that is a) molecular weight was less than 500 Da b) the number of hydrogen bond donors was not more than 5 c) the number of hydrogen bond acceptors was not more than 10 d) the calculated octanol-water partition coefficient (ClogP) was not more than 5(Lipinski *et al*., 2001). Additionally, DL was further assessed with Lipinski violations equal to or less than 1. Furthermore, OB was considered satisfactory if it was greater than or equal to 0.50. These stringent criteria were applied to filter and prioritize compounds with optimal drug-like properties for further investigation.

4.2 Compound-target Prediction and Inflammatory target identification

The Swiss Target Prediction Tool (http://swisstargetprediction.ch/), STITCH (http://stitch.embl.de/), and Similarity Ensemble Approach (SEA - https://sea.bkslab.org/) were utilised in identifying potential targets associated with the filtered compounds. The canonical SMILES of the compounds served as input for these prediction tools. Specifically, targets with a probability equal to or greater than 0.5 and TC (Tanimoto coefficient) equal to or greater than 0.5, focusing on Homo sapiens, were collected. The utilization of multiple prediction tools aimed to enhance the dependability of the predicted targets. After obtaining the predicted targets, duplicates were meticulously removed to ensure the accuracy and integrity of the dataset.

For inflammatory target identification, comprehensive databases such as GeneCards (https://www.genecards.org/) and OMIM (Online Mendelian Inheritance in Man – https://www.omim.org/), specializing in human genes and genetic disorders, were employed to extract targets connected with inflammation. The search strategy involved utilizing keywords such as “inflammation,” “inflammatory diseases,” “rheumatoid arthritis,” “dermatitis,” “atherosclerosis,” and “inflammatory bowel disease” to ensure a thorough exploration of inflammation-related targets.

To refine the focus of the project and pinpoint the most notable targets present across various areas of inflammation, an integration of Venn diagrams was employed. The Venn diagram

creator tool available online (https://bioinformatics.psb.ugent.be/webtools/Venn/) facilitated the creation of Venn diagrams for intersection analysis. By inputting the data from the searches as lists, the Venn diagrams visually represented the overlap, highlighting the target proteins common to all searches. This strategic approach aimed to streamline the project's concentrate on the most universally implicated targets in inflammation.

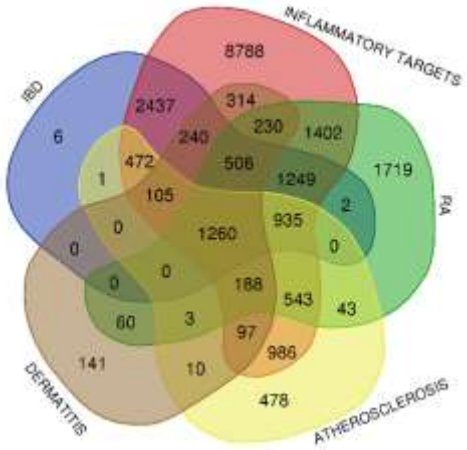

Figure 2: Venn diagram showing targets common in all inflammation related searches in databases

4.3 Network construction for compounds-target and identification of top compounds

The compounds derived from *Phyllanthusniruri*, and their associated predicted targets, were integrated to construct a comprehensive compound-target network. Cytoscape (v 3.10.1) served as the platform for this network construction, providing a user-friendly interface for visualizing and analysing complex biological networks. The components and their targets were represented as nodes and interactions between them were represented as edges.

The CytoHubba plugin was integrated into Cytoscape which facilitated the recognitionof top compounds within the network by employing a degree ranking approach. Compounds with higher degrees in the network, indicating a greater number of interactions with targets, were recognized as key compounds. These key compounds, also termed hub compounds, played a notable role in the network, suggesting their significance in modulating multiple targets.

4.4 Constructing PPI networks and identification of Common targets and top targets in inflammation

The STITCH plugin was integrated into Cytoscape (v 3.10.1). This facilitated the creation of separate PPI networks for targets connected to inflammation and those associated with compounds from *Phyllanthus niruri*. The merging of these two distinct networks resulted in the creation of a common-target network, providing a holistic view of potential intersections between plant compounds and inflammatory functions.

To understand the pivotal genes within this integrated network, the CytoHubba tool was employed. CytoHubba employs three distinct ranking algorithms—degree ranking, closeness ranking, and betweenness ranking. Degree ranking emphasizes genes with the highest number of interactions, underscoring their pivotal role in the network. Closeness ranking identifies genes in close proximity to others, indicating their significance in information flow. Lastly, betweenness ranking identifies genes acting as crucial bridges among different parts of the network, revealing their influence on communication between proteins.

The importance of these ranking algorithms aimed to pinpoint the most influential genes within the common-target network. This analysis provided valuable insights into potential top players in the intricate interplay between *Phyllanthusniruri* compounds and inflammatory pathways. The results shed light on genes with central roles, those closely connected to others, and those acting as essential bridges—critical information for understanding the molecular dynamics of the interaction.

4.5 Molecular docking

The method of molecular docking is done to analyse the interactions among the identified key targets and compounds. This critical step serves to validate the findings obtained from previous stages. Molecular docking is a predictive tool that estimates the binding affinity between the targets and compounds investigated in the study. The identified key compounds act as ligands, and their 3D structure files in SDF format were retrieved from PubChem.

To obtain target proteins from the hub genes or key targets for docking, Uniprot IDs of key genes in *Homo sapiens* were obtained from Uniport database (https://www.uniprot.org/). The Uniprot ID for target genes in Homo sapiens served as input in the Protein Data Bank (PDB) (https://www.rcsb.org/) to get the 3D protein structures. Selection criteria included choosing protein structures with X-ray diffraction values ideally less than 2.5.

Subsequently, the obtained protein structures underwent pre-processing in BIOVA Discovery studio (v21.1.0.20298), involving the removal of heteroatoms, any bounded ligands and water molecules to obtain the target proteins in their refined form for docking studies. CB-Dock 2 (https://cadd.labshare.cn/cb-dock2/php/index.php), a precise protein-ligand binding tool, was employed for the molecular docking method. This tool predicts binding sites through cavity detection and aims to get he least binding energy, for the best conformation.

The molecular docking procedure included obtaining both Vina score rankings and visualizations of the bound complexes. The stability of compound-target complexes was evaluated in accordance with their docking scores, with a preference for scores less than -5, indicating a more stable and favorable binding interaction.

4.6 Molecular dynamics (MD) simulations

Two compound-target complexes with the very least binding energy of key target were further selected for MD simulations. In our study, we employ molecular dynamics simulations to explore the stability and movements of the compound-target complexes identified in our research, offering a deeper insight into their dynamic behaviour. This approach enhances our understanding of how these interactions occur.

We employed GROMACS (v 4.6.3) and Charms-all-atom force field for generating protein topology file. The binding orientation of complex was obtained from CB dock 2 and PRODRUG tool was utilised to create ligand topology file. The solvation was executed in a triclinic box with spc216 water and neutralization was carried out by adding Na+ and Cl- ions. The complete system was then relaxed by running energy minimisation with the steepest drop. For electrostatic interactions PME algorithms were utilised and pme_order was set to 4, Fourier spacing set to 0.16 and scale was set at 299K. subsequently, using modified Berendsen thermostat, the entire system was specified for thermal equilibration for 1ns, NPT was conducted using V-rescale at 300K and temperature coupling. 50ns MD simulation was performed on each complex without any position restraints. At every 2ps the output was saved and the velocities and coordinates, nstvout and nxtout were respectively held at every 10 Ps.

For the Van der Waals and Coulombic forces, 1.0 mm cut-offs were provided. Particle Mesh Ewald (PME) and Periodic Boundary Conditions (PBC) were used in a 50 ns simulation to calculate the long-range electrostatic interaction. Following that, the MD simulation results were plotted as graphs of the Radius of Gyration (Rg), Root Mean Square Deviations (RMSD), and Root Mean Square Fluctuations (RMSF), and the resulting values were derived as g_rms, g_rmsf, and g_gyrate. (Sreenithya et al..2022)

4.7 Gene ontology and pathway analysis

Gene ontology and pathway analysis is called “enrichment analysis” and is done to analyse and unravel the complexities of biological data, we employ Gene Ontology (GO) and pathway analysis to decode the functions and interactions of genes. GO analysis categorizes genes according to their involvement in biological processes, cellular components, and molecular functions, offering a functional annotation that sheds light on gene roles. Meanwhile, pathway

analysis illuminates the intricate networks and relationships between genes, providing a holistic view of their coordinated actions within specific biological pathways. These analyses serve as invaluable tools for simplifying and interpreting vast datasets, aiding inanalysing the broader functional context of genes and their implications in health and disease.

Gene ontology and pathway analysis was conducted using online tool called SR plot (https://www.bioinformatics.com.cn/en). Keyword "GO pathway enrichment analysis" was typed in search box and the icon for GO pathway enrichment analysis is selected. the obtained top target genes were used as input and the organism was set as human. The obtained file is then downloaded after process completion into the system for analysis and interpretation.

5.RESULTS AND DISCUSSION

5.1 Plant compound collection and filtering and target prediction

A thorough search of chemical and plant databases, along with relevant literature on the phytochemistry of *Phyllanthusniruri,* produced a pool of 72 bioactive chemicals. Rigorous filtering, considering Drug Likeness (DL) and Oral Bioavailability (OB) criteria ≥ 0.50, resulted in the selection of 51 compounds meeting the defined standards. The canonical SMILES of these components were then employed in target prediction using STITCH, SEA, and the Swiss Target Prediction tool. Applying stringent conditions (Tanimoto Coefficient, TC ≥ 0.5, and Probability ≥ 0.5), we identified 413 targets associated with 28 compounds. Following the removal of duplicates, a refined list of 233 targets with Probability ≥ 0.5 and TC ≥ 0.5 was obtained.

5.2 Inflammation related target identification.

Keywords such as "inflammation", "inflammatory diseases", "rheumatoid arthritis", "arthritis", "inflammatory bowel disease", "dermatitis", and "atherosclerosis" were systematically entered into the search interfaces of GeneCards and OMIM databases. This inquiry generated over 43,000 search results specifically related to Homo Sapiens. To streamline and focus our investigation, a Venn diagram analysis was conducted, revealing 1260 common genes present in all search results.

5.3 Constructing networks and identifying common-targets and hub genes and compounds

Using Cytoscape(v 3.10.1), a compound-target network is built, this network consisted of 277 nodes and 413 edges. CytoHubba plugin was run for shortest path display using degree ranking to find 10 key compounds in which top 4 key compounds found were quercetin with rank 100, ellagic acid with rank 58, beta-sitosterol with rank 32 and kaempferol-3-rhamnoside with rank 27. These compounds degree ranking suggests that these compounds have maximum interactions

in the network. Quercetin has 100 edges, ellagic acid had 58 edges, beta-sitosterol had 32 edges and kaempferol-3-rhamnoside had 27 edges in the network.

In addition to quercetin and ellagic acid, two additional compounds from *Phyllanthus niruri* that demonstrated stable binding relationships with every protein tested were beta-sitosterol and kaempharol-3-rhamnoside. This indicates that these substances may have potential for success in reducing inflammation. Many research has drawn attention to *Phyllanthus niruri*'s bioactive components, ellagic acid and quercetin, for their possible anti-inflammatory properties (Gil et al., 2021, Aghababaei et al., 2023). Beta-sitosterol have been researched for potential anti-inflammatory and anti-oxidant effects in zebrafish and showed promising effects (Zhang et al., 2023) and kaempferol-3-rhamnoside commonly known as afzelin have been associated with potential anti-tumor and anti-oxidant effects (Masuma Akter et al., 2017). This suggest that the key compounds found from *Phyllanthus niruri* have predicted anti-inflammatory and anti-oxidant properties making it a potential candidate in incorporating into strategies for mitigating inflammatory conditions.

Similarly, a PPI network of inflammatory targets (1260) was constructed using STITCH plugging in Cytoscape this network consisted of 1102 nodes and 57769 edges. Another PPI network was created using targets that are associated with the compounds from *Phyllanthusniruri* using the similar tools this network produced 233 nodes and 2398 edges. these both PPI networks were combined, and the result was a PPI network for common targets with 90 nodes and 978 edges that are prevalent in inflammation as well as targets linked to identified drugs.
combining the CytoHubba plugin, the top 9 hub genes were determined from this combined network by combining the betweenness, degree, and closeness rankings. IL6, AKT1, NFKB1, EGFR, ESR1, PTGS2, MMP9, SRC, and JUN were these hub genes. The majority of network interactions are seen in these genes.

The major genes that we found to be important in our analysis include IL6, AKT1, NFKB1, EGFR, ESR1, PTGS2, MMP9, SRC, and JUN. These genes have a variety of important functions in mediating inflammation and causing a variety of inflammatory disorders and conditions. Central proinflammatory cytokine (IL6) has been linked to rheumatoid arthritis and other chronic inflammatory illnesses. Crucial for cell survival pathways, AKT1 exhibits links to inflammation and is linked to cancer and chronic inflammatory disorders. Inflammatory illnesses are often associated with the activation of NFKB1, a critical transcription factor that orchestrates immunological and inflammatory responses.EGFR, a protein that promotes cell division and growth, has been connected to both inflammatory skin diseases and several types of cancer. ESR1, which mediates the effects of estrogen, is studied in inflammatory illnesses connected to the joints and affects inflammatory responses. An important modulator of inflammatory

responses, PTGS2 (COX-2) is an enzyme involved in prostaglandin synthesis and a prominent target for anti-inflammatory medications. MMP9 is involved in tissue remodelling and has been linked to long-term inflammatory diseases including rheumatoid arthritis. A kinase involved in cell signalling called SRC is connected to inflammatory reactions and has been connected to cancer and a number of inflammatory illnesses. As a component of the AP-1 transcription factor, JUN controls the expression of certain genes in response to inflammation.

These targets' diverse functions in the intricate ecology of inflammation are highlighted by the designation of these genes as important ones. Finding out how *Phyllanthus niruri* compounds might affect these complex inflammatory targets will provide important information on how *Phyllanthus niruri* compounds might reduce inflammation and function as anti-inflammatory drugs.These substances have surfaced as putative targets of some *Phyllanthus niruri* chemicals, suggesting that *Phyllanthus niruri* may regulate these gene pathways. A more sophisticated approach to assessing the effects and potential of the top chemicals in *Phyllanthus niruri* will come from analysing their interactions with these genes.

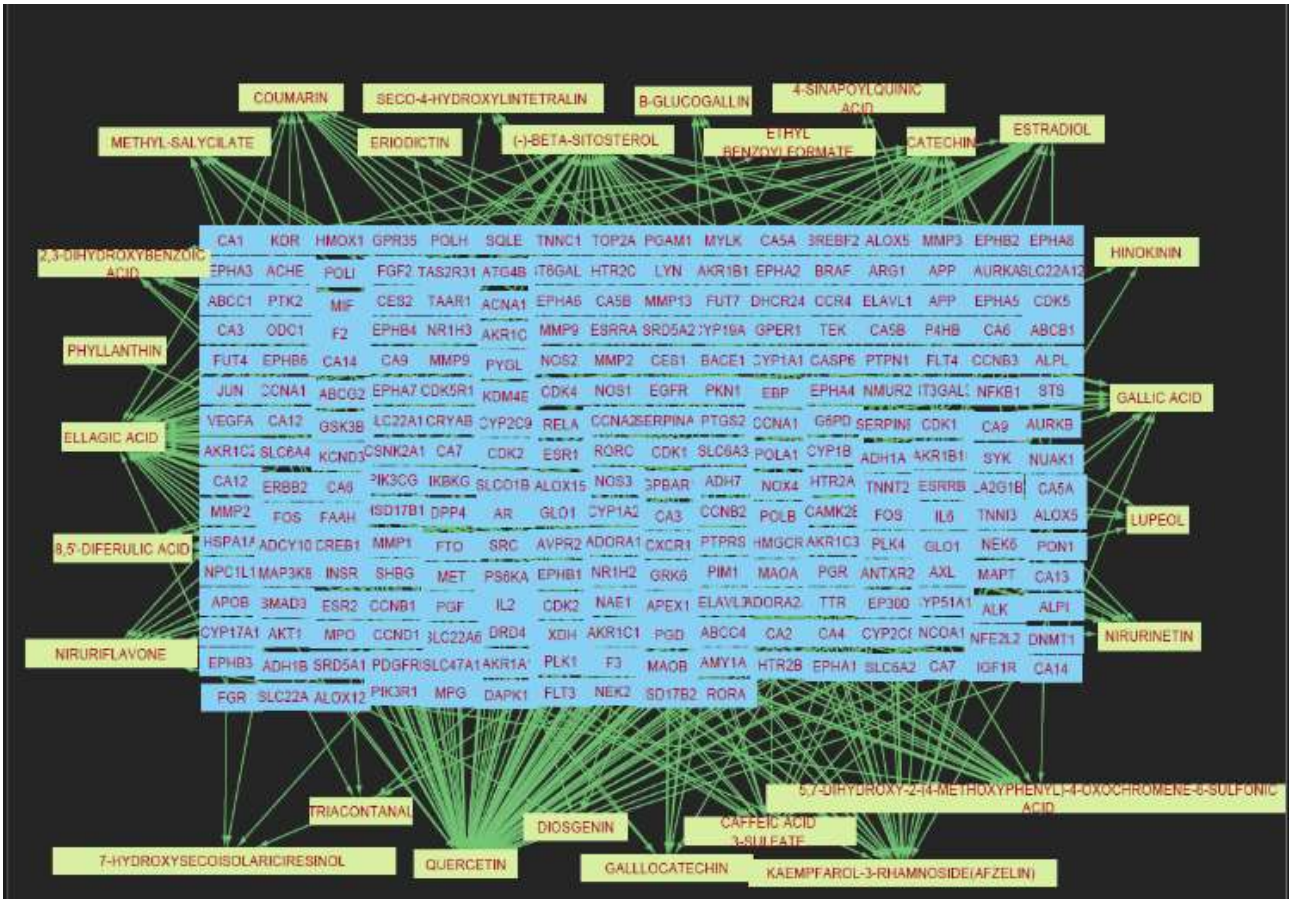

Figure 3: Network constructed using Cytoscape for compounds and their predicted targets with 277 nodes and 413 edges, compounds represented in yellow boxes and targets represented in blue boxes

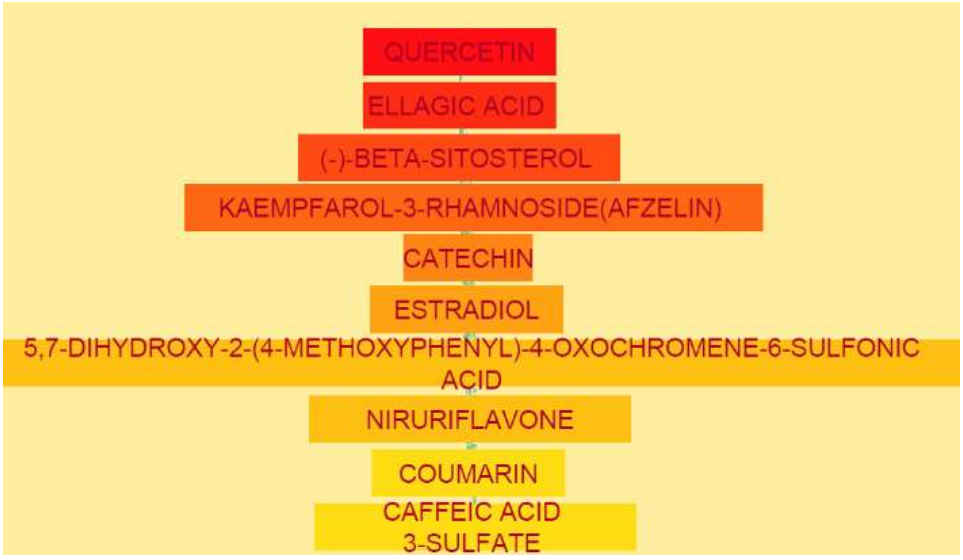

Figure 4: Top compounds in degree ranking order using CytoHubba plugin

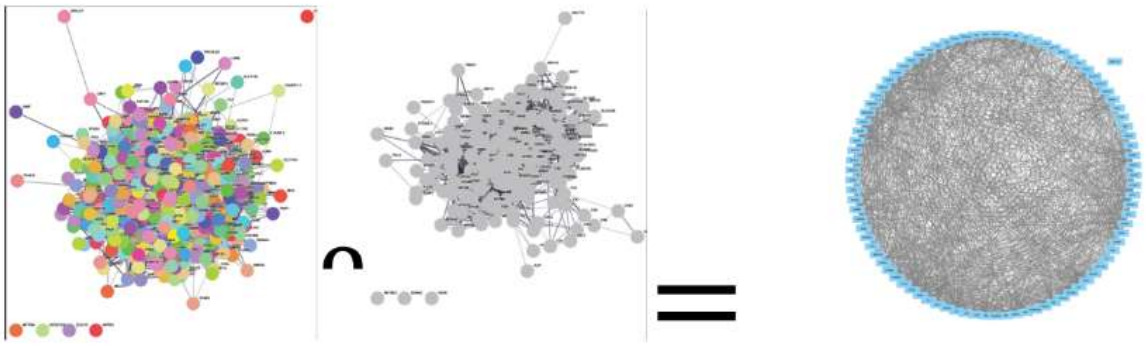

Figure 5: PPI network of inflammatory targets and PPI network of predicted targets upon merging and intersection produced PPI network consisting 90 common targets from both inflammation and predicted targets with 90 nodes and 978 edges

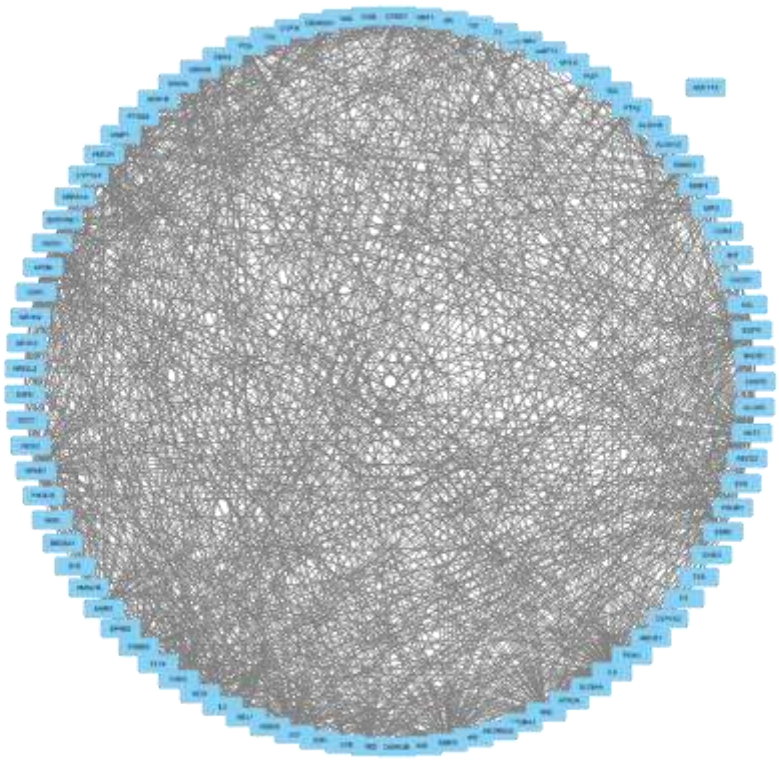

Figure 6: Merged network showing 90 nodes and 978 edges, nodes indicate the targets that are common in inflammation related targets (1260) as well as predicted targets of *Phyllanthus niruri* (233)

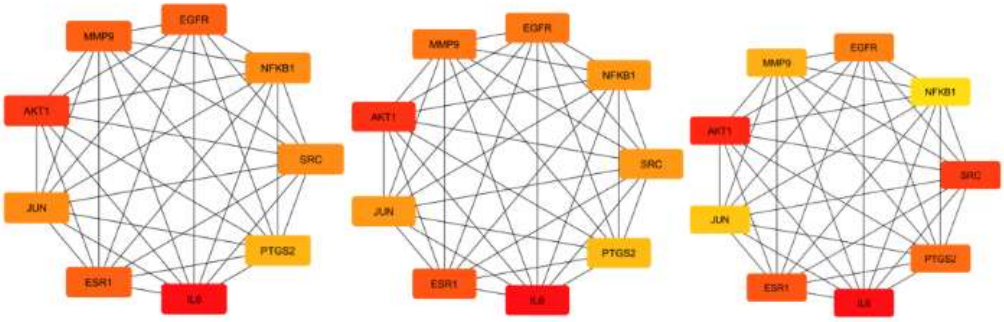

Figure 7: Hub/top/key genes identified by CytoHubba based on degree, betweenness and closeness ranking respectively from merged network

5.4 Molecular docking

CB Dock 2 was used for molecular docking in order to minimize the binding interactions between the top drugs and targets. According to the molecular docking results, all targets with substances questioned had docking scores of less than -5, indicating a stable binding relationship. Figure 8 compiles the docking results, whereas Figure 9 displays the docked complexes.

With a binding score of less than or equal to -7, quercetin, ellagic acid, beta-sitosterol, and kaempferol-3-rhamnoside each generated 5, 6, 6, and 7 complexes, respectively. This implies that the chemicals present in *Phyllanthus niruri* may play a major role in regulating inflammation and may even have anti-inflammatory properties.

With every investigated chemical, including beta-sitosterol, PTGS2 had the highest docking score, achieving a value of -10.4. As a result, PTGS2, denoted by PDB ID 5f19, became a prominent target in our investigation, exhibiting noteworthy docking scores of -9.0 with ellagic acid, -9.7 with kaempferol-3-rhamnoside, and -8.9 with quercetin. This implies that PTGS2 gene pathways and gene products may be significantly modulated by *Phyllanthus niruri.*

Phyllanthus niruri's primary target in the modulation of inflammation is PTGS2. As PTGS2 is created in response to inflammatory stimuli, it is also known as cyclooxygenase-2 (COX-2) and is a crucial enzyme implicated in inflammation. One important indicator of inflammation is elevated COX-2 expression. A number of inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel illnesses, and cancer, have been linked to PTGS2 dysregulation. PTGS2 is involved in both acute and chronic inflammatory responses. One useful strategy for developing anti-inflammatory medicines is to target PTGS2.

QUERCETIN

PDB-ID	Vina score	Cavity Size	Center			Size		
			X	Y	Z	X	Y	Z
1alu	-6.5	722	6	-24	18	21	21	21
1a07	-7.9	1571	59	-2	35	21	21	21
1gkc	-9.5	437	62	31	114	21	21	21
1gwr	-8.1	920	-3	-3	16	21	21	21
1m14	-7.8	3283	29	9	50	32	35	21
1svc	-6.8	168	52	18	29	21	21	21
1unq	-6.0	105	16	16	2	21	21	21
5f19	-8.9	4431	14	49	65	21	29	29
6y3v	-6.4	151	21	-21	-3	21	21	21

B-SITOSTEROL

PDB-ID	Vina score	Cavity Size	Center			Size		
			X	Y	Z	X	Y	Z
1alu	-6.5	436	-5	-27	7	25	25	25
1a07	-7.3	293	47	7	25	25	25	25
1gkc	-7.4	437	62	31	114	25	25	25
1gwr	-8.2	920	-3	-3	16	25	25	25
1m14	-7.5	3283	29	9	50	32	35	25
1svc	-6.8	515	39	28	47	25	25	25
1unq	-7.4	116	14	6	17	25	25	25
5f19	-10.4	4431	14	49	65	25	25	25
6y3v	-6.7	151	21	-21	-3	25	25	25

ELLAGIC ACID

PDB-ID	Vina score	Cavity Size	Center			Size		
			X	Y	Z	X	Y	Z
1alu	-7.0	166	14	-31	0	19	19	19
1a07	-7.5	643	102	26	-23	19	19	19
1gkc	-7.3	585	40	22	143	19	19	19
1gwr	-9.2	920	-3	-3	16	19	19	19
1m14	-8.0	3283	29	9	50	32	35	19
1svc	-6.2	168	52	18	29	19	19	19
1unq	-6.4	116	14	6	17	19	19	19
5f19	-9.1	26146	22	39	36	35	35	35
6y3v	-6.4	116	29	-3	-22	19	19	19

KAEMPFEROL-3-RHAMNOSIDE

PDB-ID	Vina score	Cavity Size	Center			Size		
			X	Y	Z	X	Y	Z
1alu	-6.5	166	14	-31	0	22	22	22
1a07	-8.9	482	69	4	20	22	22	22
1gkc	-8.5	437	62	31	114	22	22	22
1gwr	-6.8	3587	-4	0	-1	29	34	22
1m14	-8.4	3283	29	9	50	32	35	22
1svc	-6.4	168	52	18	29	22	22	22
1unq	-6.4	116	14	6	17	22	22	22
5f19	-9.7	26146	22	39	36	35	35	35
6y3v	-6.6	513	33	-26	2	22	22	22

Figure 8: Tables showing docking scores of top 4 compounds with 9 top targets using CB Dock 2

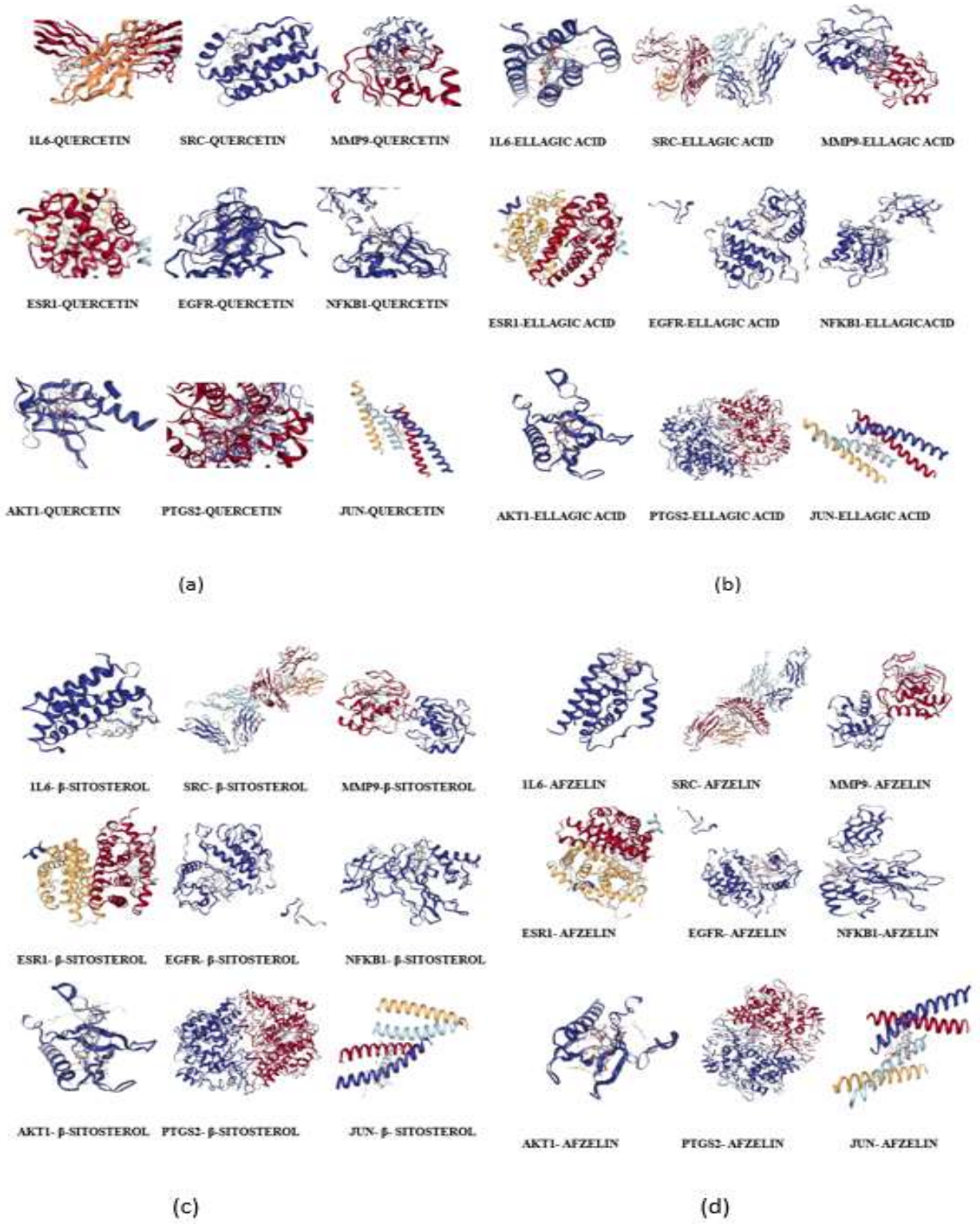

Figure 9: docked complexes with quercetin (a), docked complexes with ellagic acid (b), docked complexes with beta(β)-sitosterol (c), docked complexes with kaempferol-3-rhamnoside(afzelin) (d)

5.5 Molecular dynamics (MD) simulation

Other than ellagic acid and quercetin, beta-sitosterol and kaempferol-3-rhamnoside had the lowest docking scores in our studies. However, because these compounds were a part of a novel discovery, they were used for molecular dynamics to understand the stability of complexes over time.

To analyse the molecular dynamics and stability over time, molecular dynamics simulations were performed for the 5f19-kaempferol-3-rhamnoside complex, which had a docking score of -9.0, and the 5f19-β-sitosterol complex, which had a docking score of -10.4. For every complex, the dynamics is performed for 50 ns, and graphs for the RMSD, H-bond, gyration and RMSF are produced and analysed.

5.5.1 RMSD-Root Mean Square Deviation

RMSD or Root Mean Square deviation is the measure of deviation of the c-alpha backbone throughout the simulation. RMSD is calculated as the square root of the mean of the squared differences between corresponding atomic coordinates.

In this investigation, we used molecular dynamics simulations to examine the Root Mean Square Deviation (RMSD) profiles of two complexes, 5f19-kaempferol and 5f19-beta-sitosterol, in order to better understand their dynamic behaviour. Initial variations were seen for the 5f19-kaempferol complex, indicating that the system was adjusting to the simulation environment during the equilibration phase. After about 10 ns, RMSD values steadied at 0.2 nm, indicating that the conformation remained constant during the simulation. With only slight variations, the complex kept this stable structure and eventually attained equilibrium. On the other hand, the 5f19-beta-sitosterol complex showed a notable structural alteration as seen by an early jump in RMSD. But just like with the kaempferol complex, stability happened later, with RMSD values varying between 0.15 and 0.2 nm. Both complexes showed stability throughout the simulation, though slightly differently. While the beta-sitosterol combination saw modest conformational changes, the 5f19-kaempferol complex showed slightly less variation, suggesting better structural stiffness. Our findings emphasize the stability of both complexes and the subtle differences in their dynamic behaviour, highlighting the significance of taking particular ligand interactions into account in molecular dynamics simulations.

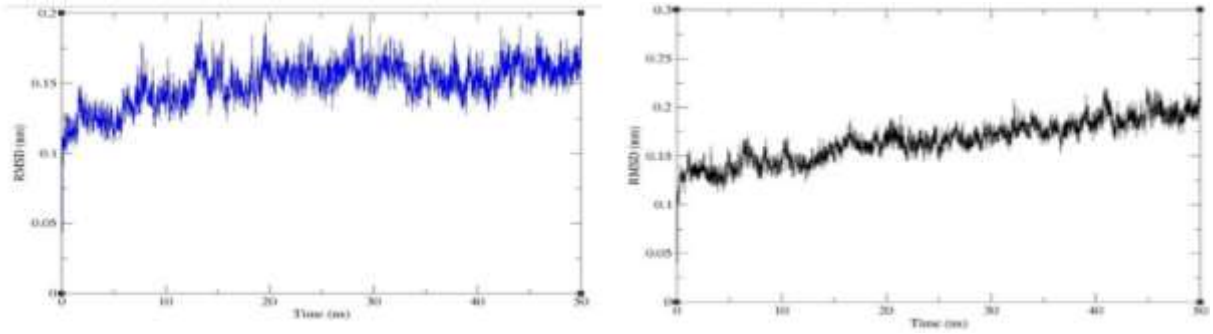

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Figure 10: RMSD graphs for 5f19-β- sitosterol**Figure 11:** RMSD graph for 5f19-kaempferol

5.5.2 H-bond

Hydrogen bonds are important links in ligand-protein interactions in molecular dynamics simulations. The 5f19-kaempferol and 5f19-beta-sitosterol complexes exhibit unique patterns that are gradually convergent, according to our findings. The hydrogen bond counts of the two complexes first fluctuate during equilibration and then stabilize, however at differing rates. After around 10 ns, the kaempferol complex attains a stable interaction pattern and keeps an average of 1.5 hydrogen bonds. In a similar vein, the hydrogen bond count of the beta-sitosterol complex first rises and then levels out in a stable range. These results highlight the strength of ligand-protein interactions, illustrating a dynamic but stable network of hydrogen bonds. All things considered, both complexes show stability, highlighting the crucial part hydrogen bonds play in regulating the kinetics of ligand-protein interaction.

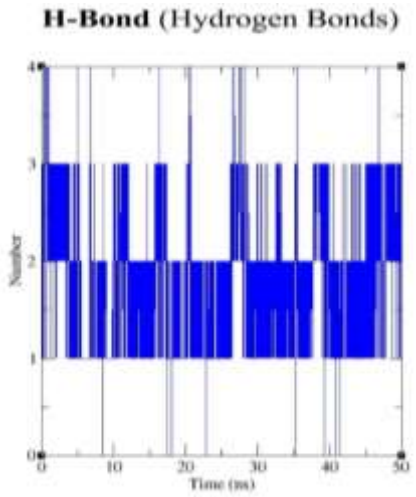

Figure 12: H-bond graph for 5f19-β-sitosterol

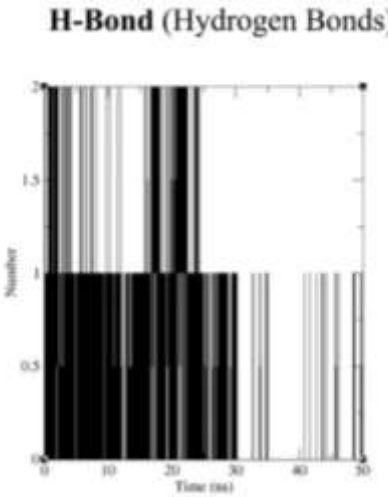

Figure 13: H-bonding graph for 5f19-kaempferol

5.5.3 Radius of Gyration

We evaluated the compactness and stability of two complexes, 5f19-kaempferol and 5f19-beta-sitosterol, using the Radius of Gyration (Rg) in our molecular dynamicssimulation. After about 10 ns, the 5f19-kaempferol complex's fast fluctuations in Rg stabilized at 3.18 nm, showing constant conformation and compactness throughout the simulation. In contrast, the Rg of the 5f19-beta-sitosterol complex increased sharply at first before stabilizing at a distance of 0.15 to 0.2 nm, indicating a stable structure with very slight variations. Remarkably, negligible conformational variations were seen in the beta-sitosterol complex, although the kaempferol complex exhibited slightly less fluctuation, indicating stronger structural stiffness. Rg values indicate the compactness and stability of both complexes, which overall retained their structural integrity.

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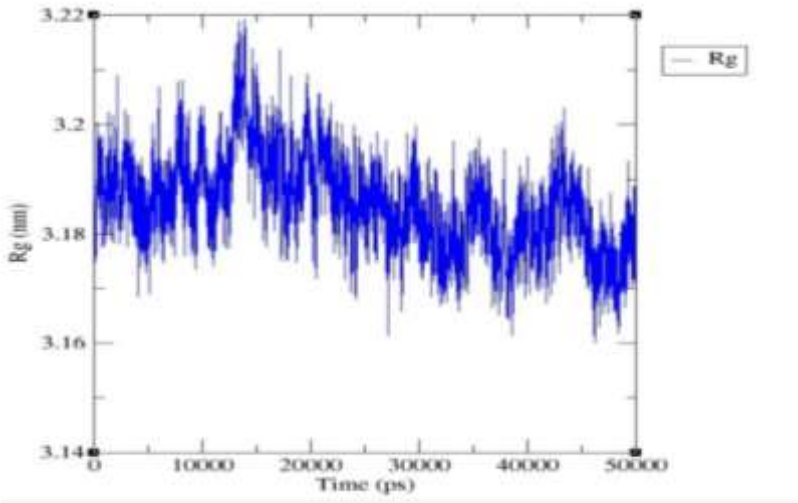

Figure 14: radius of gyration for 5f19-β-sitosterol

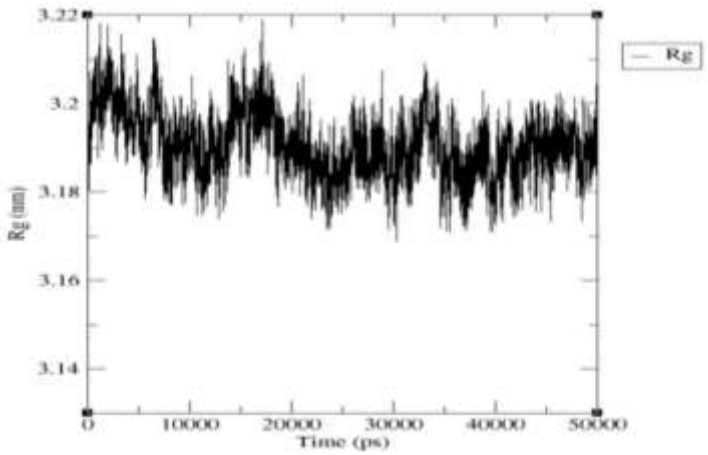

Figure 15: Radius of gyration for 5f19-kaempferol

5.5.4 RMSF- Root mean square fluctuations

Analysing the RMSF profiles for the complexes of 5f19-kaempferol and 5f19-beta-sitosterol, we found several patterns that emphasize residue flexibility over the course of the cycles. A prominent peak was found in the 5f19-kaempferol complex at residue 350, which suggests greater variations or mobility in this area. Other residues showed fewer changes, which suggests overall stability. Likewise, in the 5f19-beta-sitosterol complex, regions of greater flexibility within the complex were highlighted by prominent peaks in RMSF values at different residue locations, while other residues retained relative stability. These results highlight how dynamic protein-ligand interactions are and how crucial it is to comprehend flexibility at the residue level in order to better understand ligand binding processes and protein function. Additional investigation into these flexible areas should yield important information about the complexes' dynamic activity.

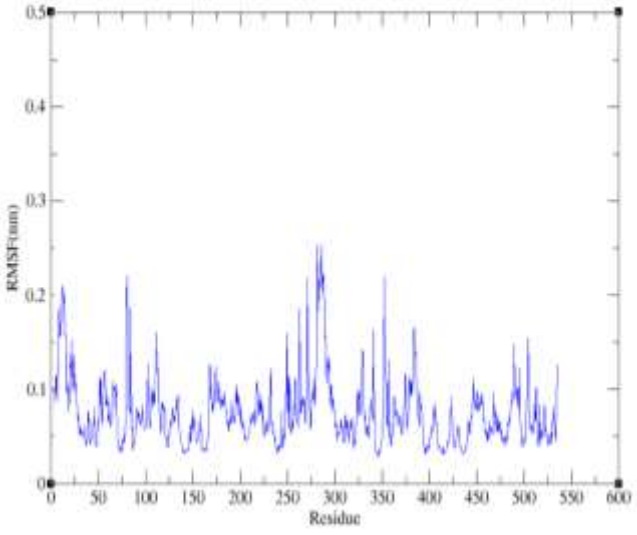

Figure 16: RMSF graph of 5f19-β-sitosterol

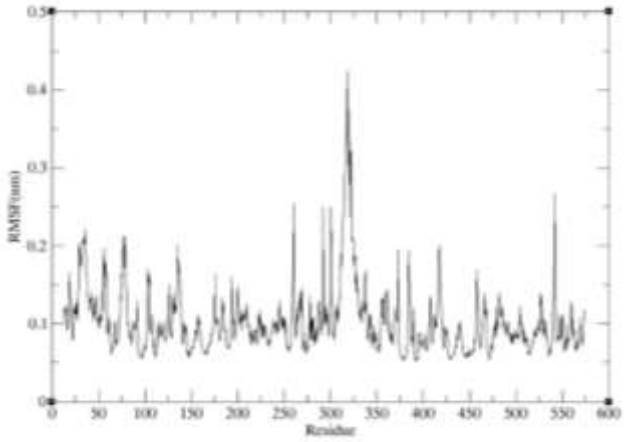

Figure 17: RMSF graph of 5f19-kaempferol

In conclusion, our thorough examination of molecular dynamics simulations for the 5f19-kaempferol and 5f19-beta-sitosterol complexes has yielded significant insights into their dynamic behavior and stability. Both complexes displayed resilience throughout the simulation, maintaining stable conformations as evidenced by consistent RMSD profiles and compact structures indicated by Rg analysis. The essential role of hydrogen bonds in facilitating ligand-protein interactions was underscored by sustained bonding networks observed in both complexes. Furthermore, the identification of flexible regions within the complexes through RMSF analysis provided valuable insights into potential conformational changes. While stability was evident in both complexes, they exhibited distinct characteristics: 5f19-kaempferol displayed a slightly more rigid structure with lower RMSD fluctuation and stable hydrogen bonds, while 5f19-beta-sitosterol showed stability with specific flexible regions, despite initial hydrogen bond fluctuations. Understanding these nuances is critical for elucidating the mechanisms of ligand binding and protein dynamics, emphasizing the importance of thorough structural analyses in molecular dynamics simulations for advancing our understanding of biological processes and potential therapeutic applications. However, an extended simulation of 100ns could provide better and comprehensive idea.

5.6 Gene ontology and pathway analysis

5.6.1 GO: Biological Processes

Numerous biological processes that are considerably enriched have been identified using gene ontology enrichment analysis. These processes include the proliferation of smooth muscle cells, their response to chemical stimuli, reactive oxygen species (GO:0000302), and neuro-inflammatory reactions. These results imply that *Phyllanthus niruri*'s constituents may have a regulatory function in inflammatory situations, which are frequently marked by the presence of reactive oxygen species.

The enrichment of smooth muscle cell proliferation-related gene ontology (GO:0048661) suggests that drugs derived from *Phyllanthus niruri* may slow down the development of atherosclerosis by focusing on genes involved in this process. According to study, smooth muscle cell proliferation is necessary for the advancement of atherosclerotic illness (Lim S et al., 2014).

It is well recognized that chemical stressors actively contribute to inflammation by initiating a number of signalling pathways that result in the generation of cytokines and chemokines, two important inflammatory mediators (GO:0062197). The relationship between *Phyllanthus niruri* and cell proliferation also raises the possibility of a regulatory impact on tumors like leiomyosarcomas, which are frequently found in organs including the uterus, stomach, and intestines and are characterized by muscle cell proliferation.

While these interpretations are encouraging, it is vital to remember that they should be backed up by experimental data. Research indicates that *Phyllanthus niruri* demonstrates cytotoxic properties against multiple cancer cell lines and has the potential to amplify the cytotoxic effects of doxorubicin against cell lines that express breast cancer (Ola E. Abdel-Sattar et al., 2023). Furthermore, *Phyllanthus niruri* has been found to possess hepatoprotective, hypolipidemic, and anti-inflammatory qualities, all of which may enhance its potential as a treatment for cancer and atherosclerosis (Muhammad Farrukh Nisar et al., 2018).

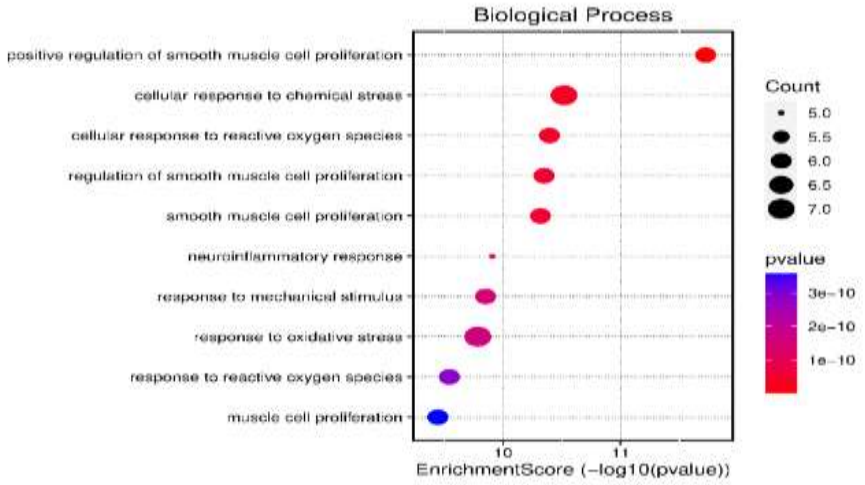

Figure 18: Dot-plot showing gene ontology enrichment of biological processes

5.6.2 GO: Molecular functions

The molecular functions linked to the regulation of inflammatory responses, such as ATPase binding (GO:0051117), estrogen receptor binding (GO:0030331), and RNA polymerase II-specific DNA-binding transcription factor binding (GO:0061629), were significantly enriched in our analysis.

- **ATPase Binding (GO:0051117)**: The enrichment of ATPase binding implies that the gene products could impact biological functions that depend on ATP hydrolysis, like immune cell activation and muscular contraction, therefore influencing the inflammatory response.
- **Estrogen Receptor Binding (GO:0030331)**: The intricate nature of hormone signalling in inflammation is highlighted by the interaction with estrogen receptors, which suggests a function in regulating gene expression patterns that may have pro- or anti-inflammatory effects.
- **RNA Polymerase II-specific DNA-binding Transcription Factor Binding (GO:0061629)**: Since these transcription factors are necessary for the expression of genes involved in the immune response, their function highlights the significance of transcriptional control in inflammation.

Furthermore, the identification of Protein kinase C binding (GO:0005080) highlighted the significance of signalling in the immune response and inflammation. The molecular functions enriched in our study provide insights into the complex regulatory mechanisms at play in inflammation and offer potential targets for therapeutic intervention.

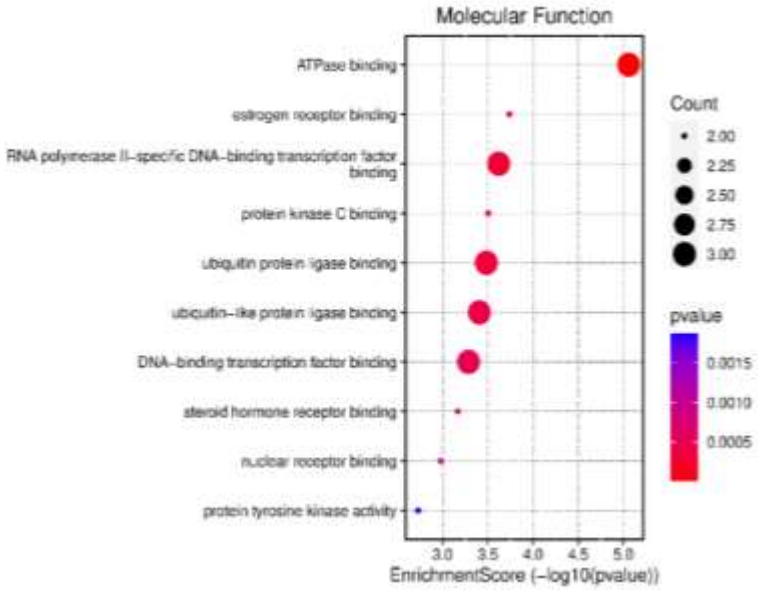

Figure 19: Dot-plot showing molecular function gene ontology enrichment

5.6.3 GO: Cellular components

As shown in figure 18, the cellular components that were enriched in our analysis were mostly the membrane raft (GO:0045121), membrane microdomain (GO:0098857), and membrane region (GO:0098589). This indicates that the majority of the activity of the gene products occurs in the membrane region, where they bind to membrane-resident receptors to regulate.

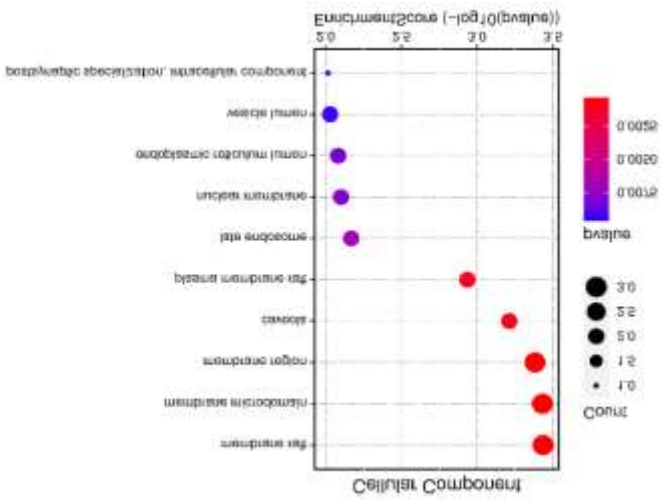

Figure 20: Dot-plot showing cellular component gene ontology enrichment

5.6.4 Pathway analysis

The most enriched pathway, according to the pathway analysis, is the endocrine resistance pathway. As demonstrated by studies by Musheyev D and Alayev A, this pathway is closely linked to estrogen resistance positive breast cancer, suggesting that controlling it may be a useful strategy for controlling the disease. This suggests that *Phyllanthus niruri* chemicals may modulate the expression of breast cancer-related genes.

The complicated and nuanced interaction between inflammation and cancer may have contributed to the enrichment of the pathways related to breast cancer in our study. According to a study by DeNardo DG, the biological components of the immune system interact intricately and have a major impact on the pathogenesis of breast cancer. It has been demonstrated that acute immune responses, especially those mediated by cytolytic T-lymphocytes, offer protection against the start of tumor formation. On the other hand, tumor growth and progression have been linked to the persistent stimulation of humoral immunity, Th2 cell infiltration, and the existence of innate inflammatory cells with a protumor polarization.

These results emphasize the potential of innate and adaptive leukocytes as therapeutic targets by underlining their dualistic nature in the setting of breast carcinogenesis. The *Phyllanthus niruri* compound's anti-inflammatory qualities may be a viable method for influencing these immune pathways, enhancing the pathways linked to breast cancer and possibly slowing the progression of the disease. Furthermore, A study by Ola E. Abdel Sattar and colleagues in 2023 found that

Phyllanthus niruri compounds have been investigated for their chemo toxic and cytotoxic effect on resistant breast cancer. The results showed that *Phyllanthus niruri* in combination with DOX is an effective chemo modulatory agent against resistance breast cancer.

These findings are further supported by the complementarity of the estrogen signalling route and the endocrine resistance pathway. This may serve as a foundation for future investigations into the application of *Phyllanthus niruri* as an adjuvant therapy in the management of breast cancer, particularly when hormone resistance is a problem.

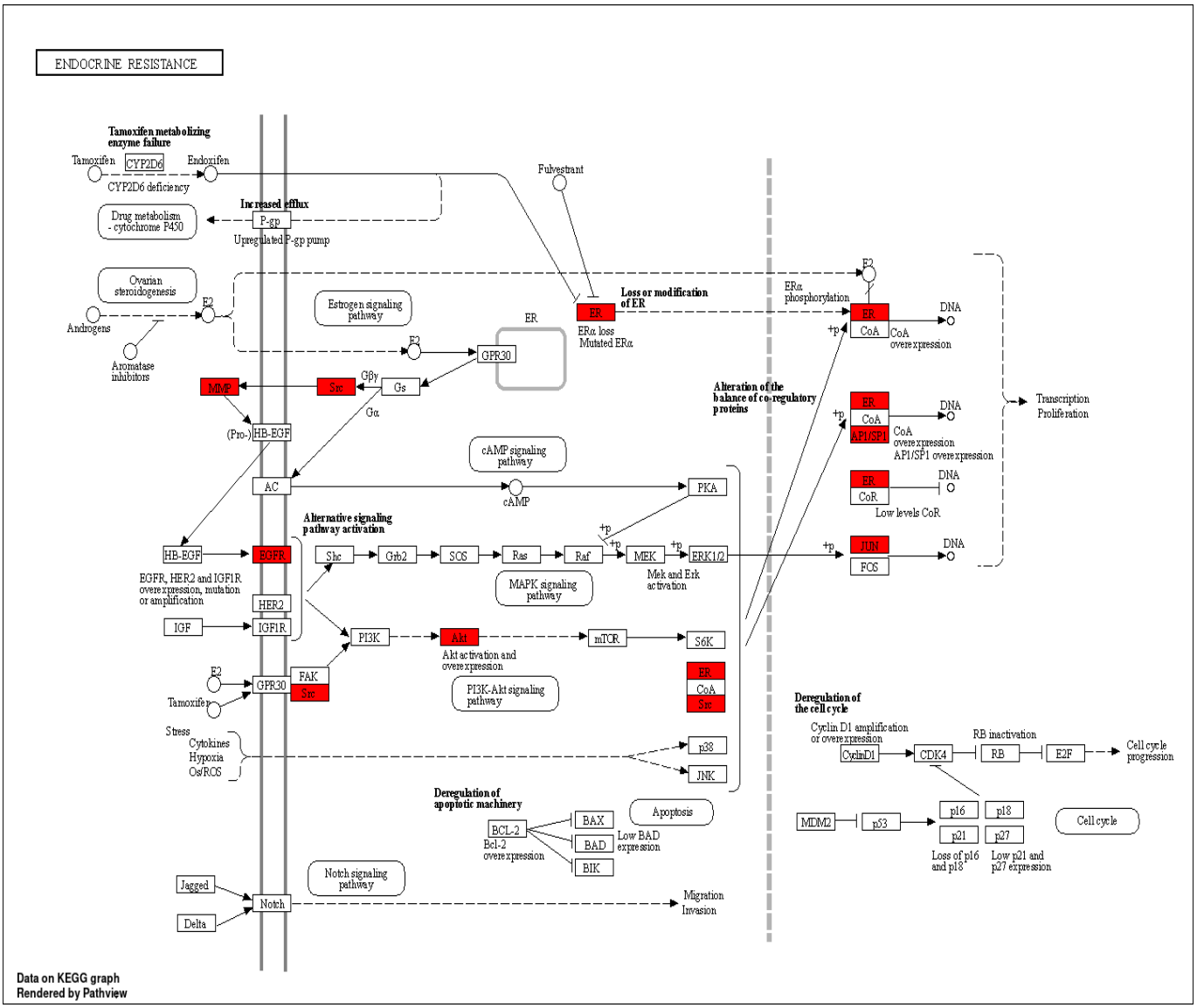

Figure 21: Endocrine resistance pathway with target genes marked in red

Phyllanthus niruri may be able to demonstrate its anti-inflammatory and immunomodulatory properties by focusing on further enriched pathways, such as the C-type lectin receptor signalling pathways, which are actively implicated in both inflammation and the immune response. When it is dysregulated, it may contribute to inflammatory illnesses by causing the synthesis of inflammatory mediators (Drouin M et al., 2020).

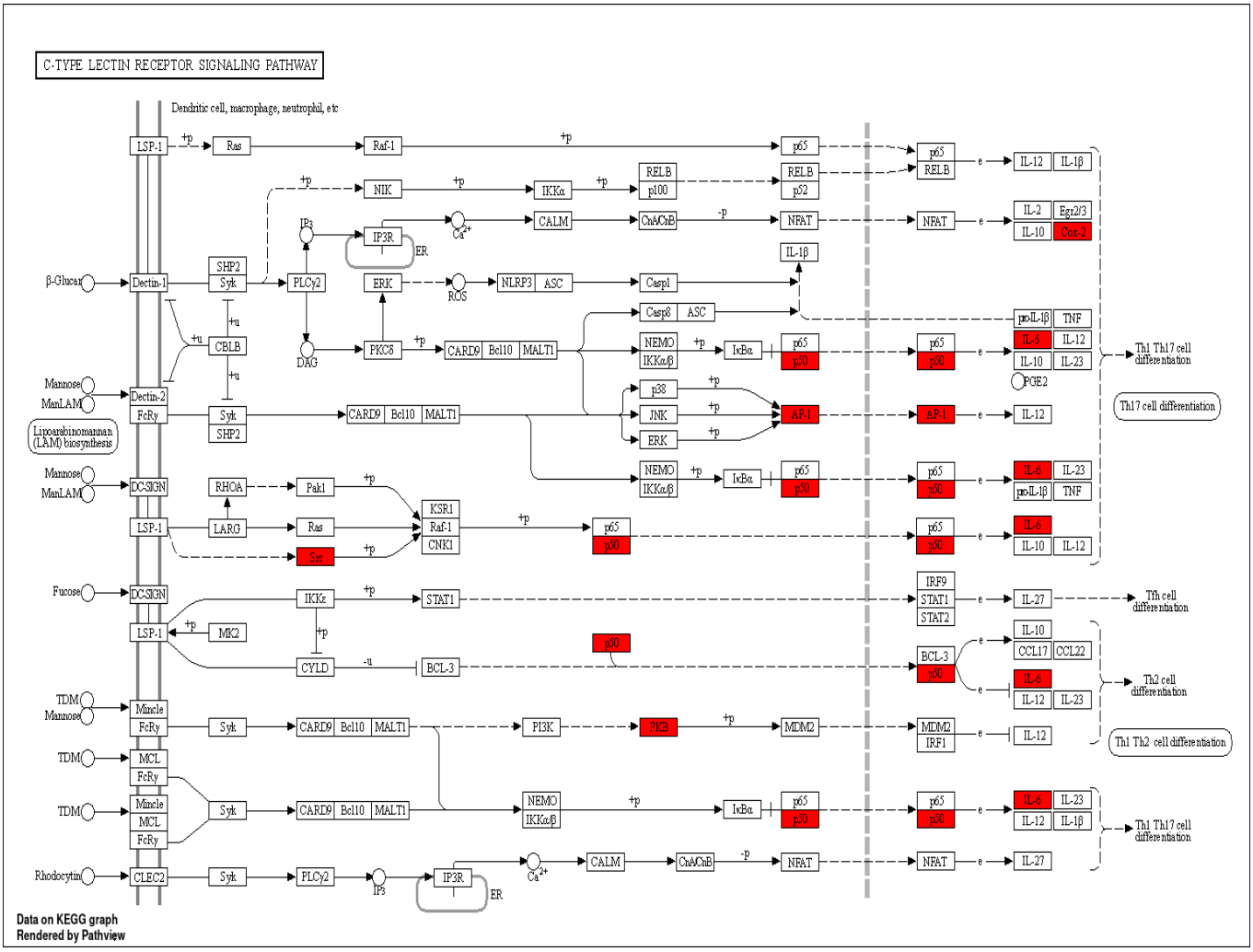

Figure 22: C-type lectin receptor signalling pathway

Enrichment of TNF signalling pathway, lipid and atherosclerosis as well as IL-17 signalling pathway could possibly suggest the immunomodulatory and anti-inflammatory activities of components from *Phyllanthus niruri* by targeting specific genes in these pathways for modulating inflammatory conditions

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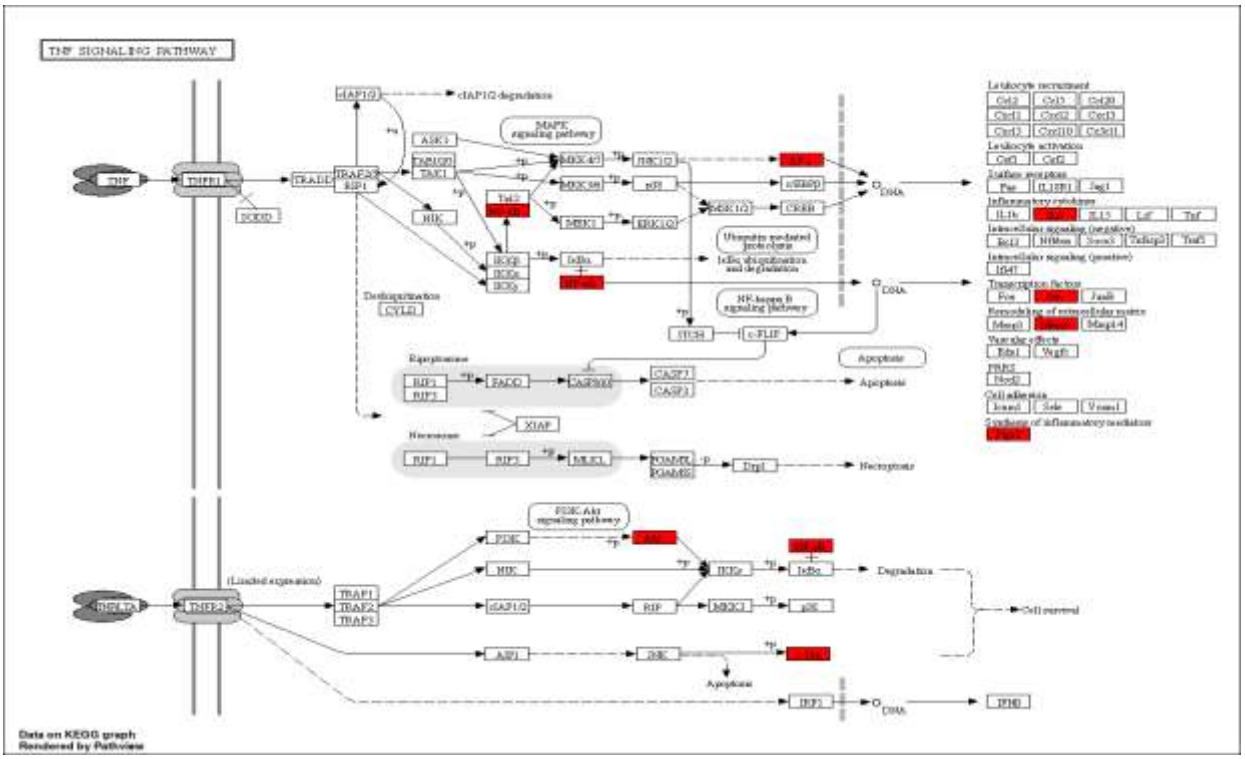

Figure 23: TNF signalling pathway

Anti-inflammatory treatments primarily target the control of the TNF signalling system, which is essential in mediating inflammatory reactions. It has been demonstrated that compounds from *Phyllanthus niruri* contain anti-inflammatory qualities, which may have an impact on this pathway.

Compounds from *Phyllanthus niruri* may interact with different elements of the TNF signalling pathway in order to produce their desired effects. As an example, they might prevent TNFαfrom initiating the NF-κB pathway, a key mediator of inflammation. These substances may lessen the expression of inflammatory mediators and pro-inflammatory cytokines by blocking NF-κB activation.

Compounds from *Phyllanthus niruri* may also alter the activity of other proteins and enzymes that are part of the TNF signalling cascade, including the IKK complex, TAK1/TAB 2/3, and cIAPs/BIRC2/3. These interactions may contribute to the reported anti-inflammatory benefits by reducing the inflammatory response.

Studies have demonstrated that Phyllanthus amarus, a closely related species to *Phyllanthus niruri*, can block the TNF signalling pathway as well as the NF-κB, MAPK, and PI3K-Akt signalling pathways in LPS-induced human macrophages (Harikrishnan et al., 2018). This implies that chemicals derived from *Phyllanthus niruri* may have comparable effects.

6. CONCLUSISON

According to our network pharmacology investigation, ***Phyllanthus niruri*** possesses a strong mix of chemicals that have noteworthy anti-inflammatory characteristics. This supports the claims made about the plant's anti-inflammatory effects. The substances, which include kaempferol-3-rhamnoside, quercetin, ellagic acid, and beta-sitosterol, have shown promise in binding with important inflammatory mediators, including EGFR, ESR1, PTGS2, NFKB1, MMP9, JUN, and SRC. Interestingly, PTGS2, or COX-2, became a key target; dynamic and molecular docking investigations indicated that the **β-sitosterol** as well as **Kaempferol-3-rhamnoside**complex might stabilize this protein and hence have anti-inflammatory or immunomodulatory effects.

Our research also suggests that ***Phyllanthus niruri*** influences genes related to oxidative stress and cell proliferation, which may have therapeutic benefits for conditions including atherosclerosis and some types of cancer. ***Phyllanthus niruri***'s target genes interact intriguingly with pathways linked to breast cancer, indicating a potential use case for this plant in breast cancer therapy approaches.

Phyllanthus niruri's important effects on lipid metabolism and atherosclerosis, TNF signalling, and IL-17 signalling pathways are also highlighted by the analysis, highlighting the plant's function in controlling inflammatory reactions. These discoveries open up new avenues for investigating the therapeutic uses of ***Phyllanthus niruri*** and its components, which may provide patients with inflammatory illnesses with hope again.

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