RESEARCH PAPER

Exploring Molecular Targets and Mechanisms of Apigenin in the Treatment of Diabetic Wound Healing by Network Pharmacology and Molecular Docking Analysis

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ABSTRACT

Background: Diabetic wounds (DW) represent a condition associated with diabetes. They are challenging to restore, and existing therapeutic alternatives are restricted. This study utilized a network pharmacology approach to examine the therapeutic potential of Apigenin (API) for the treatment of DW.

Methods: Apigenin-related targets were gathered from SwissTargetPrediction, STITCH, and GeneCards, while diabetic wound-related targets were gathered from OMIM and GeneCards. Overlapping targets were determined using Venny 2.1.0. A protein-protein interaction (PPI) network was generated using STRING and visualized using Cytoscape to determine hub targets. Gene Ontology (GO) and KEGG pathway enrichment analysis was conducted using ShinyGO (FDR < 0.05). Molecular docking was performed to determine the binding affinity of apigenin against IL-6 (PDB: 4NI7) and TNF-α (PDB: 2AZ5), followed by molecular dynamics simulation to determine the stability of the complexes.

Results: A total of 514 overlapping targets were found between apigenin and diabetic wound-related genes. PPI analysis showed 482 nodes and 16114 edges, signifying a high degree of target interconnectivity. GO and KEGG enrichment analysis showed the significant role of inflammation, with the AGE-RAGE pathway being a prominent pathway. Apigenin showed binding affinities of -6.4 kcal/mol (IL-6) and -7.4 kcal/mol (TNF-α). Molecular dynamics simulation analysis showed the stability of ligand-protein complexes with similar RMSD/RMSF values.

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Conclusion: The present study systematically proved the pharmacological targets and mechanisms of API in the treatment of DW. The current study provides important insights into the theoretical basis for DW treatment with API.

Keywords: apigenin, diabetic wound, network pharmacology, enrichment analysis, molecular docking, molecular simulation, AGE-RAGE pathway, IL-6, TNF-α.

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Introduction

Diabetes mellitus is linked to a number of alterations in the cardiovascular system, both macrovascular and microvascular, which can lead to a wide variety of problems. A significant portion of the approximately 537 million people globally living with diabetes will experience diabetic wounds at some point in their lives; of those, 19% to 34% will develop diabetic wounds at some point; 20% will need amputation of a limb as a result of diabetic wounds; and 10% will die within one year of their initial diagnosis of diabetic wounds [1-3]. This underscores the urgent necessity for innovative therapy approaches that may proficiently address the fundamental pathophysiological mechanisms implicated in DW repair. Natural compounds have garnered heightened interest for their potential in addressing complex disorders, including DWs, owing to their multi-target pharmacological characteristics and comparatively low toxicity. Apigenin (API), also known as 4′,5,7-trihydroxyflavone, is a flavonoid found naturally in a wide variety of foods, including celery, beans, fruits, and tea leaves. Among the several physicochemical features that set Api apart is the presence of 4′ hydroxyl groups at positions 5 and 7, in addition to a C2C3 double bond [4]. Additionally, AP lessens oxidative stress, decreases levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and inhibits mitochondria-mediated neuron death. It also downregulates the TLR4/NF-B signalling pathway. The results of the immunofluorescence assay demonstrated that AP inhibits the ACN-induced

activation and nuclear transfer of NF-κB [5]. Apigenin treatment can be explored as a potential method for the treatment of diabetic wounds because of its anti-inflammatory, anti-infective, antioxidant, and pro-angiogenic actions, as well as its ability to re-epithelialize diabetic wounds.

Network pharmacology, a novel methodology that combines systems biology and bioinformatics, facilitates the identification of potential drug targets and the clarification of intricate biological networks implicated in disease mechanisms, providing a thorough framework to explore the complex molecular networks and interactions that underlie drug actions [6]. By creating and analyzing a network of interactions between API and its associated molecular targets, we may attain a thorough understanding of the various paths by which API may influence DW repair, thereby uncovering its diverse therapeutic potential. Molecular docking elucidates the binding affinity of drug-target interactions, facilitating the prediction of efficacy and selectivity at the molecular level. Molecular dynamics (MD) simulations provide a dynamic view of these interactions at the atomic scale. MD simulations are essential for evaluating the stability, conformational flexibility, and binding free energy of molecule-target complexes over time, providing crucial insights into the durability and specificity of these interactions under physiological conditions [7,8]. While network pharmacology and molecular dynamics simulations have been utilized to examine various flavonoids in relation to diabetic complications, their integrated application in exploring API's function in DWs has yet to be investigated. Moreover, although certain studies have investigated the effects of API on overall wound healing, a significant study deficiency exists about its specific molecular targets, binding kinetics, and pathways related to diabetic wound healing. This study seeks to fill this gap by employing network pharmacology, molecular docking, molecular dynamics simulation, and experimental validation to thoroughly examine the therapeutic potential and mechanisms of action of API in DWs. We aim to elucidate the multi-target mechanisms of API by establishing AGE-RAGE signaling pathway in

diabetic complications, identifying key genes, and assessing enriched biological processes and pathways. Molecular docking was utilized to assess the initial binding affinity of the active pharmaceutical ingredient (API) to critical targets, while molecular dynamics (MD) simulations offered insights into the temporal stability and dynamic behavior of these interactions, thereby ensuring a comprehensive evaluation of API's molecular interactions. This work enhances the existing information on flavonoid-based therapies and establishes a basis for future clinical research on API as a potential treatment for diabetic wounds.

Experimental

Collection of the target genes of Apigenin

The researchers gathered Apigenin target information from three public databases which include SwissTarget Prediction and STITCH and Genecards [9,10]. The database targets which were acquired from the database were merged together while all duplicate entries were eliminated.

Collection of Diabetic-wound related genes

The search for diabetic wound related targets was conducted at OMIM and Genecards using the search term "Diabetic wound" [11,12]. The research team merged all target genes from different databases after they removed duplicate entries which resulted in a list of target genes for further study.

Screening of intersection targets

The researchers used Venny 2.1.0 software to determine which molecular targets Apigenin shares with diabetic wound treatment [13]. The research team examined the overlapping targets because they wanted to understand the biological functions of these targets. The study created a network visualization which showed how the compound interacted with its common targets and the disease context for their functional relationships.

Constructing Protein-protein interaction (PPI) network and screening hub genes

The study aims to develop a protein-protein interaction (PPI) network which will help identify essential genes in the research process. The common targets of Apigenin and diabetic wound were imported into the String database 12.0 to construct the protein- protein interaction network

[14]. The study required Homo sapiens as the only species with a minimum interaction score of 0.4 (medium confidence) to be used as the study's threshold. The researchers exported interaction data in TSV format to use it in network analysis with Cytoscape 3.10.3 software [10]. The CytoHubba plug-in was used to identify key targets based on the Degree algorithm [15].

GO and KEGG pathway enrichment analysis

The researchers used Gene Ontology (GO) functional annotations and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (FDR < 0.05) to study signaling pathways and biological processes and molecular functions and cellular components of Apigenin and diabetic wound core targets. The researchers used ShinyGO to perform pathway enrichment analysis [16].

Molecular Docking Using AutoDock Tools

Molecular docking analyses were carried out using the AutoDock 4.2 software package to assess the binding interactions between the ligands and target proteins. The grid boxes were set up to encompass the active binding regions of the target proteins. The docking scores were calculated in terms of binding energy (kcal/mol), which reflected the binding affinities of the ligands to their target proteins. The binding energy scores were used to determine the binding affinities of the ligands to their target proteins [17].

Molecular dynamics simulations

TNFα (2az5) and IL6 (4ni7) was shortlisted for performing MD simulation based upon their docking score and observed chemical interactions with the ligand Apigenin. MD simulation was executed for the macromolecular complex of 2az5 and 4ni7 with complexed Apigenin for a time period of 100 ns by using Desmond module of Schrodinger's Maestro software [17,18]. Moreover, incorporation of explicit solvent molecules, then neutralized by the addition of corresponding ions. The steepest-descent approach was employed to relax the system and resolve any steric conflicts or suboptimal connections among atoms to decrease the system's energy. The system was equilibrated using a brief series of low-temperature simulations at constant pressure. Positional limitations are imposed on the system alongside a gradual elevation in temperature. This increases the probability that the system will attain a stable,

balanced state before the simulation. To achieve the desired outcomes, the simulation is conducted for 100 ns, considering the system's energies, atomic locations, and RMSD values. This facilitates understanding of the system's dynamic behavior and offers long-term insights into the complexity of its structure and functional stability [19].

Results and Discussion

Identification of potential target of Apigenin and Diabetic wound

To identify the targets of Apigenin in Diabetic wound research, 640 Apigenin targets were obtained from SwissTarget Prediction and STITCH and Genecards. We gathered 6575 Diabetic wound-related targets through OMIM and Genecards to discover potential new treatment targets for Diabetic wounds. Venny 2.1.0 software revealed 514 shared protein targets through graphical analysis of drug-related and disease-related protein targets (Fig. 1).

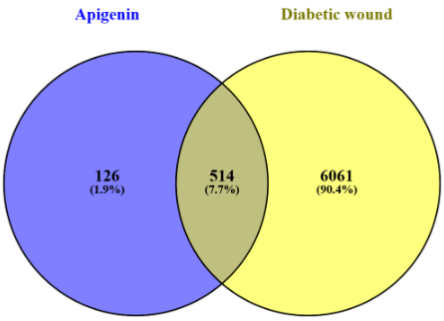

Fig. 1: Venn diagram of Apigenin targets and Diabetic wound targets.

PPI network and screening hub targets

The protein-protein interaction network was constructed by importing 514 common targets into the string database which restricted species access to Homo sapiens (Fig. 2a). The PPI network displayed 482 nodes and 16114 edges after disconnected nodes had been eliminated, while showing an average node degree of 66.9 and a PPI enrichment p-value of < 1.0e-16. The interaction data obtained from string were further imported into cytoscape for network visualization and topological analysis (Fig. 2b). The top five hub targets were identified through Degree scoring by using cytoHubba (Fig. 2c).

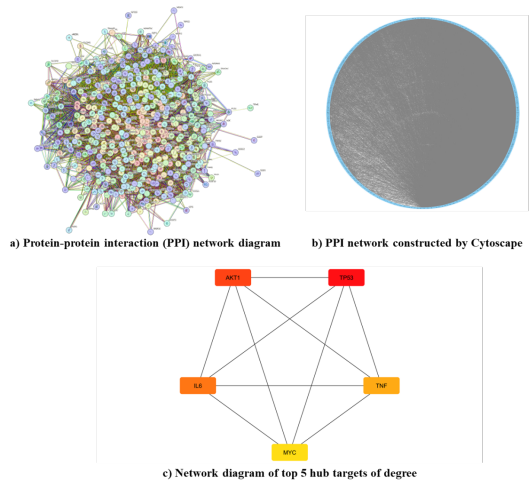

Fig. 2: Integrated protein–protein interaction (PPI) network analysis, depicting STRING-based interaction mapping, Cytoscape-mediated network visualization, and identification of the top five hub genes

GO and KEGG analysis

The potential functions of Apigenin against Diabetic wound treatment were studied through analysis of GO enrichment and KEGG pathway enrichment which examined the top five hub targets. The analysis used ShinyGO database to conduct analysis based on FDR < 0.05 adjusted statistical significance. The study found 1152 GO terms through which researchers identified 1000 biological process (BP) terms and 108 molecular function (MF) terms and 44 cellular component (CC) terms. Researchers plotted the top 10 most significant terms for biological process (BP) and molecular function (MF) while only 2 cellular component (CC) terms reached the significance threshold (Fig 3). The BP analysis included regulation of glial cell proliferation, glial cell proliferation, negative regulation of cell development, positive regulation of DNA metabolic process, response to decreased oxygen levels, regulation of DNA metabolic process, post-transcriptional regulation of gene expression, negative regulation of apoptotic process, negative regulation of programmed cell death, DNA metabolic process. The CC analysis were primarily concentrated in Interleukin-6 receptor complex, Transcription repressor complex. The MF results indicated enrichment in Eukaryotic initiation factor eif2 binding, 14-3-3 protein binding, Core promoter sequence-specific DNA binding, Protease binding, Dna-binding transcription repressor activity RNA polymerase ii-specific, Cytokine activity, Dna-binding transcription repressor activity, Molecular function activator activity, Identical protein binding, Molecular function regulator activity.

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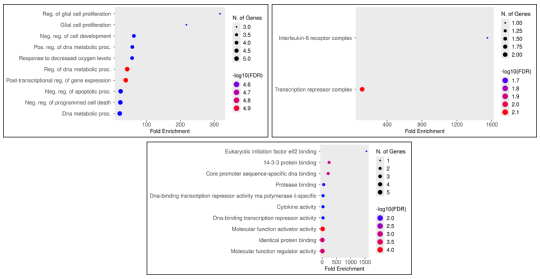

Fig. 3: GO analysis (BP, CC, MF) for target genes of Apigenin against Diabetic wound.

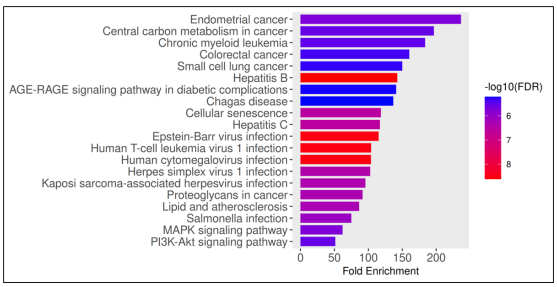

Fig. 4: KEGG pathway analysis for target genes of Apigenin against Diabetic wound.

A total of 151 KEGG enriched pathways were identified and top 20 KEGG signaling pathways were plotted. (Fig 4) The KEGG pathway analysis showed that the targets mainly focused on the following areas Endometrial cancer, Central carbon metabolism in cancer, Chronic myeloid leukemia, Colorectal cancer, Small cell lung cancer, Hepatitis B, AGE-RAGE signaling pathway in diabetic complications, Chagas disease, Cellular senescence, Hepatitis C, Epstein-Barr virus infection, Human T-cell leukemia virus 1 infection, Human cytomegalovirus infection, Herpes simplex virus 1 infection, Kaposi sarcoma-associated herpesvirus infection, Proteoglycans in cancer, Lipid and atherosclerosis, Salmonella infection, MAPK signaling pathway, PI3K-Akt signaling pathway.

The AGE-RAGE signaling pathway (hsa04933, FDR value = 5.9E-06) functioned as the main regulatory pathway which controlled diabetic complications. The AGE-RAGE signaling pathway map displays red rectangles which indicate the study's identified genes (Fig 5). The main genes which this pathway contained included AKT1 and IL6 and TNF, which researchers must use to choose important genes for their experiments.

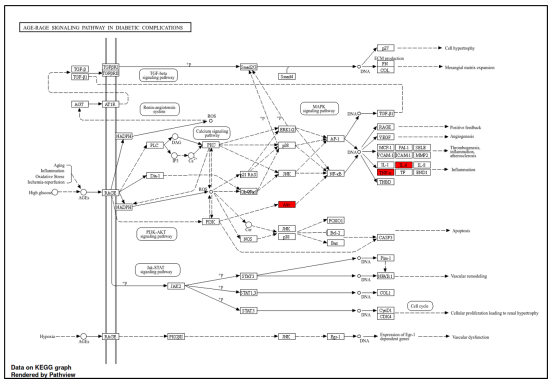

Fig. 5: The enrichment results included in the AGE-RAGE signaling pathway in diabetic complications map.

Molecular docking

Molecular docking studies also reinforced the anti-inflammatory property of apigenin in the context of diabetic wound healing, where chronic inflammation due to the action of cytokines like IL-6 and TNF-α plays a major role in the delayed healing of wounds. Apigenin showed binding affinity of −6.4 kcal/mol with IL-6 (PDB code: 4NI7) and −7.4 kcal/mol with TNF-α (PDB code: 2AZ5), suggesting strong ligand-protein interactions, especially with TNF-α. Interaction study showed that apigenin established major stabilizing interactions in the binding pocket of IL-6, including traditional hydrogen bonding and van der Waals interactions, along with hydrophobic stabilization by π-cation and π-alkyl interactions with residues ARG16, ARG24, GLN17, GLN28, LEU19, PRO18, and LYS27 (Table 1).

Table 1: Binding energies after Molecular docking interaction

Ligand	IL6 (4ni7)	TNFα (2az5)
Apigenin	-6.4	-7.4

Furthermore, in the TNF-α complex, apigenin showed high binding stabilization by hydrogen bonding (notably TYR151) and hydrophobic aromatic interactions like π-π stacked and amide-π stacked interactions, which involve residues TYR59, GLY121, LEU120, TYR119, SER60, and GLN61. These biological interactions indicate that apigenin can efficiently bind and stabilize the active binding sites of these inflammatory cytokines, thereby potentially inhibiting the cytokine-mediated signaling pathways (Fig. 6,7). Likewise, in an LPS-induced inflammation model in male Wistar rats, Apigenin significantly decreased serum levels of TNF-α, IL-1β, and IL-6 in a dose and time-dependent manner [20]. Also, pretreatment with apigenin markedly attenuated inflammatory cell infiltration, suppressed NF-κB activation, reduced pro-inflammatory cytokines, enhanced IL-10 production, and restored oxidative balance in spleen tissue [21]. Additionally, comparative in vitro findings revealed that apigenin, kaempferol, and resveratrol effectively reduced TNF-α secretion [22]. As high levels of IL-6 and TNF-α are known to be correlated with the chronic phase of inflammation, impaired angiogenesis, and delayed re-epithelialization in diabetic wounds, the high binding and favorable

interaction properties of apigenin indicate its potential utility as an effective therapeutic agent for promoting diabetic wound healing by regulating inflammatory pathways.

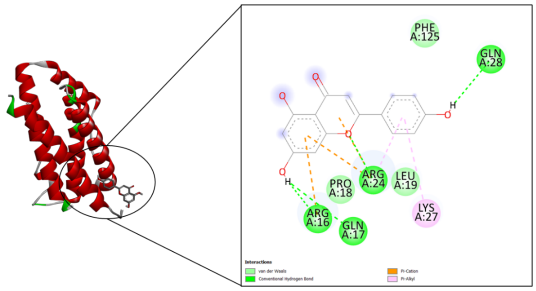

Figure 6 2D and 3D representation of Apigenin docked with IL-6 receptor protein (PDB ID: 4ni7)

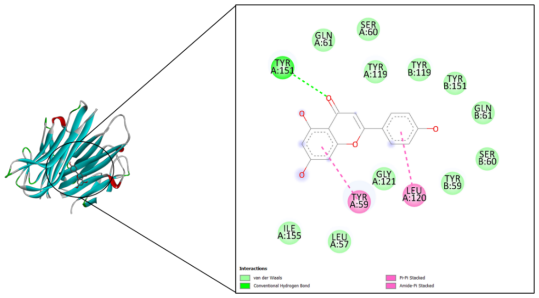

Figure 7 2D and 3D representation of Apigenin docked with TNFα receptor protein (PDB ID: 2az5)

Molecular Docking (MD) Simulation

Simulation of IL6 (4NI7) receptor complex with ligand Apigenin: The molecular dynamics (MD) simulation of the apigenin-IL-6 complex further supported the stability of the docked conformation and the retention of important interactions during the simulation time. The protein-ligand RMSD plot showed that the complex reached a state of equilibrium after the initial oscillations, suggesting that apigenin was properly bound to the IL-6 binding pocket throughout the simulation. The ligand RMSD values also retained an acceptable pattern, suggesting little deviation and consistent conformational stability of apigenin during binding. Moreover, the RMSF plot showed low residue fluctuations for most areas of the IL-6 protein, suggesting that the binding of apigenin did not cause significant structural instability, except for the small peaks corresponding to the flexible loops. The protein-ligand contact profile revealed that apigenin was able to sustain interactions with key residues like ARG16, LYS27, GLN28, TRP31, GLU110, ARG113, ALA114, and VAL121, which included hydrogen bonding, hydrophobic interactions, ionic interactions, and water-bridge mediated binding. The presence of sustained hydrogen bonding and hydrophobic interactions during the simulation period emphasizes the strong binding affinity of apigenin with IL-6. In conclusion, the above MD simulation results strongly validate the molecular

docking study and emphasize that apigenin is able to form a stable complex with IL-6, which may help in the suppression of IL-6 mediated inflammatory responses and thereby facilitate tissue repair and regeneration in diabetic wound healing (Fig. 8).

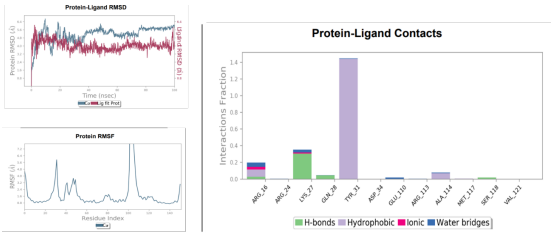

Fig. 8: Molecular Docking (MD) Simulation of IL6 (4NI7) receptor complex with ligand Apigenin

Simulation of TNF-α (2AZ5) receptor complex with ligand Apigenin: The molecular dynamics (MD) simulation of the apigenin-TNF-α complex further validated the accuracy of the docking analysis, thereby confirming the high binding affinity of apigenin to TNF-α, a critical cytokine involved in chronic inflammation during the healing of diabetic wounds. The RMSD analysis of the protein-ligand complex indicated an initial fluctuation phase followed by a stabilization phase, thereby confirming that the complex had reached equilibrium. The ligand RMSD values were also found to be within acceptable limits after the equilibration process, thereby confirming that apigenin had a stable binding orientation within the TNF-α binding pocket. The RMSF plot indicated that there was little fluctuation in the residues of the TNF-α protein, thereby confirming that the protein was rigid. The small peaks in the plot indicated the presence of flexible loops, which are usually more dynamic. Notably, the protein-ligand contact analysis showed that there were constant contacts between apigenin and several important residues, such as LEU57, ALA58, TYR59, SER60, GLN61, TYR119, LEU120, GLY121, GLU122, and TYR151, which were mediated by hydrogen bonding, hydrophobic, and water-bridge interactions. The preponderance of hydrophobic and hydrogen bond contacts indicates that the ligand binding is strong and that the interaction is stable. In general, the results of the MD simulation study indicate that apigenin has the potential to form a stable complex with TNF-α, which may be a potential inhibitor of TNF-α-induced inflammatory responses, thus validating its therapeutic use in enhancing tissue repair and healing of diabetic wounds (Fig. 9).

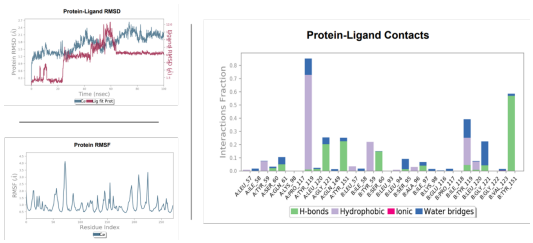

Fig. 9: Molecular Docking (MD) Simulation of TNF-α (2AZ5) receptor complex with ligand Apigenin

Discussion

Diabetic wound healing is a complex and chronic process that is often compromised by the presence of chronic inflammation, oxidative stress, hypoxia, decreased angiogenesis, and impaired tissue remodeling. In diabetic patients, wounds are often known to fail to transition from the inflammatory stage to the proliferative and remodeling stages. Cytokines such as IL-6 and TNF-alpha are major players in the chronic process of inflammation and are strongly linked to the impairment of epithelialization, collagen synthesis, and granulation tissue formation. It is, therefore, important to identify compounds that can modulate these targets of inflammation.

In the current research, a network pharmacology approach was used to investigate the potential mechanism of apigenin in the healing of diabetic wounds. A total of 640 targets related to apigenin were gathered from the SwissTargetPrediction, STITCH, and GeneCards databases, and 6575 targets related to diabetic wounds were collected from the OMIM and GeneCards databases. The intersection analysis revealed 514 common targets, which suggested a significant overlap and that apigenin could potentially affect multiple disease-related proteins. This is especially important in the context of diabetic wound healing, where multiple molecular defects occur simultaneously. Protein-protein interaction (PPI) analysis of the 514 common targets using the STRING database showed a highly interconnected network with 482 nodes and 16114 edges, along with a significant enrichment p-value (<1.0e−16). The network was further analyzed in Cytoscape, and hub gene screening revealed the top key regulatory targets, suggesting that apigenin could potentially interact with the central proteins involved in the progression of diabetic wounds.

The GO enrichment analysis further supported the relevance of the target genes identified in the diabetic wound pathophysiology. The cellular component analysis showed enrichment in the

IL-6 receptor complex, suggesting the involvement of inflammatory cytokine signaling. The molecular function analysis showed enrichment in cytokine activity and regulatory protein binding, suggesting that apigenin could potentially interact with inflammatory mediators and transcriptional regulation mechanisms.

KEGG pathway enrichment analysis revealed 151 pathways, and among them, the AGE-RAGE signaling pathway in diabetic complications was found to be one of the most significant. AGE-RAGE signaling has been reported to induce oxidative stress and the secretion of inflammatory cytokines, leading to impaired healing in diabetic wounds. The crucial genes AKT1, IL6, and TNF were found to be involved in this pathway, thus validating the potential of these genes as therapeutic targets for apigenin.

Molecular docking studies further confirmed the binding of apigenin with inflammatory cytokines. The binding affinities of apigenin with IL-6 (4NI7) and TNF-α (2AZ5) were found to be −6.4 kcal/mol and −7.4 kcal/mol, respectively, suggesting a strong binding affinity, especially with TNF-α. Apigenin was found to form stable bonds with the residues ARG16, ARG24, GLN17, GLN28, LEU19, PRO18, and LYS27 of IL-6, whereas with TNF-α, it formed stable bonds with residues TYR151, TYR59, LEU120, GLY121, TYR119, SER60, and GLN61, thus validating its anti-inflammatory properties

Molecular dynamics simulation further supported the stability of complexes. RMSD and RMSF analysis revealed that apigenin was strongly bound to both IL-6 and TNF-α throughout the simulation process, with stable hydrogen bonding, hydrophobic interactions, and water-bridge interactions. These results further support the docking analysis and illustrate the robustness of apigenin binding in dynamic conditions.

In conclusion, the present study suggests that apigenin could facilitate diabetic wound healing by targeting various pathways and molecules, especially inflammatory cytokines and AGE-RAGE signaling. Although these computational predictions are encouraging, experimental verification is needed to validate the role of apigenin in wound contraction, collagen deposition, angiogenesis, and cytokine regulation in diabetic wounds.

Conclusion

The strategy adopted in the current study was an integrated approach involving network pharmacology, molecular docking, and molecular dynamics simulation to investigate the potential therapeutic use of apigenin in the healing of diabetic wounds. The result of the network pharmacology analysis revealed a substantial overlap between the targets of apigenin and diabetic wounds, which indicated the multitarget pharmacological characteristics of apigenin. The protein-protein interaction network and hub gene analysis further supported the involvement of key regulatory proteins, suggesting that apigenin has the potential to influence critical molecular mechanisms associated with chronic inflammation and tissue repair. The enrichment analysis using GO and KEGG pathways demonstrated that the targets identified were significantly enriched in biological processes and pathways that are closely related to the pathogenesis of diabetic wounds, particularly the AGE-RAGE signaling pathway, as well as inflammation and survival pathways such as PI3K-AKT, Jak-STAT, Calcium and MAPK signaling pathways. Furthermore, molecular docking analysis confirmed the high binding affinity of apigenin with the major pro-inflammatory cytokines IL-6 and TNF-α, and interaction analysis indicated the presence of stable hydrogen bonding and hydrophobic interactions with the major amino acid residues. In addition, molecular dynamics simulation analysis confirmed the stability of the apigenin-protein complexes, with no structural changes or fluctuations during the simulation period. In conclusion, the findings of the present study strongly suggest that apigenin may be a promising therapeutic agent for the healing of diabetic wounds by targeting multiple sites associated with the inflammatory response and complications of diabetes. However, it is essential that the proposed mechanisms of action be validated in vitro and in vivo to confirm their relevance to the acceleration of wound healing in diabetic patients.

Acknowledgements

N/A

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Table 1: Binding energies after Molecular docking interaction

Ligand	**IL6 (4ni7)**	**TNFα (2az5)**
Apigenin	-6.4	-7.4

Figure Captions

Fig. 1: Venn diagram of Apigenin targets and Diabetic wound targets.

Fig. 2: Integrated protein–protein interaction (PPI) network analysis, depicting

Fig. 3: GO analysis (BP, CC, MF) for target genes of Apigenin against Diabetic wound.

Fig. 4: KEGG pathway analysis for target genes of Apigenin against Diabetic wound.

Fig. 5: The enrichment results included in the AGE-RAGE signaling pathway in diabetic complications map.

Figure 6 2D and 3D representation of Apigenin docked with IL-6 receptor protein (PDB ID: 4ni7)

Figure 7 2D and 3D representation of Apigenin docked with TNFα receptor protein (PDB ID: 2az5)

Fig. 8: Molecular Docking (MD) Simulation of IL6 (4NI7) receptor complex with ligand Apigenin

Fig. 9: Molecular Docking (MD) Simulation of TNF-α (2AZ5) receptor complex with ligand Apigenin