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RESEARCH ARTICLE

Unlocking the molecular targets of ursolic acid for diabetic wound healing

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Abstract

Diabetic wounds, which account for 85% of non-traumatic amputations due to necrosis and gangrene, are a leading cause of hospital admissions among diabetic complications. These wounds require protective layers against infection and bioactive materials like ursolic acid (UA) to promote healing processes. This study investigates the molecular targets of UA through bioinformatics and *in silico* analysis to inhibit diabetic wounds. After obtaining an online database of the genes related to diabetic wound healing, the genes influenced by UA were identified using a Venn diagram. Subsequently, the top 10 target proteins associated with molecular pathways were scrutinized using CytoHubba to elucidate their roles. Finally, a molecular docking study was performed on TP53 and TNF for further investigation. UA orchestrates molecular cascades involving apoptosis and pyroptosis-related genes in many types of cells such as fibroblasts, keratinocytes, endothelial, and erythrocytes. This study is expected to promote the development of UA-containing natural products as wound healing agents for diabetic patients. This study may also support *in vitro* and *in vivo* studies of UA and UA-containing natural products in diabetic wound healing activities.

Keywords: diabetic, molecular pathway, natural product, skin, tissue regeneration

INTRODUCTION

The skin, the largest organ of the body, protects the body from external factors such as bacteria, chemicals, physicals, and temperature. As the first line of defense, it is constantly exposed to potential injury (Takeo et al., 2015). Disruption or damage to the integrity of the skin or organ tissue, making the body vulnerable to pain and infection, is defined as a wound, which can be classified as either chronic or acute wound (Kujath & Michelsen, 2008; Irfan-Maqsood et al., 2016; Nagle et al., 2023; Herman & Bardoni, 2023). Acute wounds are caused by environmental factors involving traumatic injury and have the potential to heal relatively quickly and efficiently, in contrast to chronic wounds, which stem from metabolic imbalances and lead to prolonged healing times. It has imbalanced production and degradation of cells and extracellular matrix (ECM), such as collagen. Chronic wounds such as venous/vascular ulcers, diabetic ulcers, and pressure ulcers, exhibited imbalanced production and degradation of cells and extracellular matrix (ECM), such as collagen (Kujath & Michelsen, 2008; Irfan-Maqsood et al., 2016; Dasari et al., 2021).

Diabetic wounds/ulcers are chronic wounds caused by diabetic conditions (Irfan-Maqsood et al., 2016). The main mechanism underlying these wounds involves compromised immune function and neuropathic conditions in diabetic patients. Minor skin injuries may go unnoticed due to neuropathy and a weakened immune system, leading to the development of chronic wounds as the body fails to prevent infections. Diabetic wounds exhibit a prolonged inflammatory phase of healing compared to wounds in non-diabetic patients. (Irfan-Maqsood et al., 2016; Kujath & Michelsen, 2008; Dasari et al., 2021). These wounds can develop into ulcers, typically in the areas of the foot that encounter repetitive trauma and pressure (Oliver & Mutluoglu, 2023; Wang et al., 2022). Diabetic foot ulcers contribute to more admissions than any other diabetic

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complication. Overall, about 15 – 25% of patients with diabetes mellitus develop foot ulcers and 1% ultimately require amputation. Presently, diabetes accounts for 85% of non-traumatic amputations (Bakri et al., 2011; Oliver & Mutluoglu, 2023; Syauta et al., 2021). Patients with diabetes mellitus also develop severe atherosclerosis of the small blood vessels in the legs and feet, resulting in compromised blood flow, which further exacerbates diabetic foot infections. The inability of blood to reach the wound delays the healing process, eventually leading to necrosis and gangrene (Oliver & Mutluoglu, 2023; Syauta et al., 2021).

The process of wound healing is intricate and involves a sophisticated interaction among various types of cells, cytokines, mediators, and the vascular system, encompassing 4 main stages: hemostasis, inflammation, new tissue formation, and remodeling (Setiawati et al., 2024; Wallace et al., 2023; Gonzalez et al., 2016). During the inflammatory phase of normal wound healing, initial phagocytic macrophages (M1) induce inflammation. These are then replaced by M2 macrophages, which promote angiogenesis, contribute to the synthesis of extracellular matrix (ECM), and have anti-inflammatory properties. However, in diabetic wounds, the transition from inflammatory macrophages to anti-inflammatory macrophages is hindered, leading to an overproduction of proinflammatory cytokines by macrophages (Gonzalez et al., 2016; Kujath & Michelsen, 2008; Dasari et al., 2021; Takeo et al., 2015). Therefore, diabetic wound healing treatment is more challenging than normal wound healing.

Traditional commercial wound dressings for diabetic wounds not only require a protection layer against microbial infection but also require bioactive materials or drugs to aid in wound treatment or stimulate the wound healing phases. Moreover, active wound dressings containing bioactive materials are more efficient in cell restoration and proper healing of chronic wounds (Ali et al., 2023; Alven et al., 2022; Da Silva et al., 2023). One promising active compound embedded into wound dressing is ursolic acid (UA). UA is a triterpenoid compound found in various parts of plants and exhibits diverse pharmaceutical properties and therapeutic effects. UA-containing plants have been used as herbal medicine, such as Pearl grass (*Hedyotis corymbosa* (L.) Lam.) (Anam et al., 2017), *Clinopodium revolutum* (Lamiaceae) (Huaman et al., 2021), and *Anredera cordifolia* (Ten.) (Hanafiah et al., 2021). They exhibited excellent pharmacological activities, including blood glucose reduction, antioxidation, and anti-inflammation, and have been used to treat diabetic wounds (Alam et al., 2021; Lv et al., 2022; Kartini et al., 2021). UA demonstrates a protective effect on liver injury in diabetic mice by regulating lipid metabolism, reducing oxidative stress, and enhancing the ability of anti-oxidation in the liver. UA also ameliorates ulcerative colitis by regulating intestinal microbiota and inflammatory cell infiltration. Compared to those expensive growth factors and refractory oxide, UA showed some unique advantages including low cost, low toxicity, as well as muti-target functions (Lv et al., 2022). Despite numerous studies investigating UA as a promising compound for wound healing, no study has yet explored its molecular targets based on bioinformatics data and *in silico* tests.

This study aimed to investigate the molecular pathways of UA through bioinformatics and *in silico* testing to inhibit diabetic wounds. By employing bioinformatics, the gene targets of UA in the diabetic wound healing pathway were identified. Furthermore, protein-ligand docking and simulation techniques were utilized to identify novel medications for diabetic wound healing. Additionally, a predicted molecular pathway inhibition was constructed based on gene targets and previous studies.

MATERIALS AND METHODS

Data mining and collection

NCBI (www.ncbi.nlm.nih.gov), OMIM (www.omim .org), and GeneCard (www.genecard.org) were used to mine data on proteins and genes related to diabetes and wound healing. Direct protein targets of ursolic acid were mined from www.stitch.embl.de, and indirect protein targets were obtained from www.string-db.org. Direct and indirect proteins were combined to determine the intersection of genes affected by ursolic acid in diabetes and wound healing using a Venn diagram (www.interactivenn.net). The affected genes included both upregulated and downregulated genes.

Protein-protein interaction (PPI) network and gene clustering construction

The Protein-Protein Interaction (PPI) network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins STRING-DB v11.5 (www.string-db.org version 10.5). STRING was employed to identify neighboring interactors for each protein node, with a confidence score threshold of ≥ 0.90 to ensure high reliability of the interactions, derived from experimental data and curated databases. The resulting PPI network was visualized using Cytoscape software 3.9.1 (https://cyto scape.org/), allowing for analysis of the network's structural properties. For gene clustering within the

PPI network, a graph-based clustering approach considering the network topology was implemented. This MCODE algorithm was used to identify densely connected regions within the PPI network, focusing on local neighborhood density optimization. The MCC and DMNC algorithms provided by the Cyto-Hubba plugin were utilized to identify the top 15 genes, classifying them as hub genes. A predicted molecular cascade of ursolic acid (UA) in diabetic and wound healing of the skin was drawn using Biorender (https://app.biorender.com/). The bioinformatics analysis was conducted on a computer with an Intel(R) Core (TM) i3-1005G1, CPU@1.20GHz, RAM 4GB.

Molecular docking

From the MCC result, several genes influencing diabetic wound healing, such as TP53 and TNF α, were identified. TP53 (8SWJ) and TNF α (2AZ5) were obtained from (www.rcsb.org) in .pdb file format. Ligands were separated and protonated using BIOVIA Discovery Studio 2021, with ligands protonated using Gaisteiger charges and protein using Kollman charges. The ligand was placed at the coordinates x = -5.649, y = -12.450, z = -2.836 for TP53 and x = -19.163, y = 74.451, z = 33.837 for TNF α. The macromolecule and the ligand were saved in pdbqt. format using AutodockTools 1.5.7. Docking was conducted within 30 x 30 x 30 grid with spacing points 0,375 Å. using the Genetic algorithm with GA runs set to 100. The method was validated by calculating the Root Mean Square Deviation (RMSD) value of the control ligand before and after re-docking, with an RMSD value ≤2.00 Å. RMSD values were calculated using LigRMSD (https://ligrmsd.appsbio.utalca.cl/). Cross-docking was performed with UA as a ligand obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). The ligand was prepared and docked using the same method as the control ligand. Docking results were obtained in dlg format and the pose with the lowest binding energy was complexed with the macromolecule and stored in pdbqt format. Docking results were visualized using BIOVIA Discovery Studio 2021 in 3D and surface models, and protein-ligand interactions were verified by determining interaction fingerprints (IFPs) with geometric rules. Moreover, docking results were visualized using BIOVIA Discovery Studio 2021 to observe protein-ligand interactions and calculate the binding energy.

RESULTS

Protein-protein interaction (PPI) network and gene clustering construction

Ursolic acid (UA) is a lipophilic pentacyclic triterpenoid compound found in plants, particularly abundant in fruits and vegetables (Figure 1a). Through bioinformatic analysis, UA has 72 genes involved in diabetic and wound healing from a total of 18,807 diabetic genes and 6,642 of wound healing genes (Figure 1b). These 72 genes exhibit extensive interactions from the protein-protein interaction (PPI) network analysis (Figure 1c) with a PPI enrichment p-value <1.0e-16. The top 15 ranks of MCC and DMNC algorithms for gene cluster analysis were gained using Cytoscape (Figure 2). The top 10 MCC genes play crucial roles in diabetic and wound healing, including Tumor protein p53 (TP53), B-cell lymphoma 2 (BCL2), Caspase-3 (CASP3), B-cell lymphoma 2 like 1 (BCL2L1), Caspase-8 (CASP8), Myeloid cell leukemia-1 (MCL1), Tumor Necrosis Factor (TNF), Poly (ADP-ribose) polymerase 1 (PARP1), Caspase-9 (CASP9), Cytochrome C, Somatic (CYCS) (Figure 2a). All affected genes by UA include upregulated (TP53, CASP3, CASP8, TNF, PARP1, CASP9, CYCS) and downregulated (BCL2, BCL2L1, MCL1) genes.

Furthermore, we explored the role of the top 10 MCC genes by UA in diabetic wound healing pathways (Table 1). Some target genes such as TP53, CASP3, and TNF, directly affect cell apoptosis. Other genes, such as BCL2, BCL2L1, and MCL1 are involved in preventing cell apoptosis and inducing cell pyroptosis. Apoptosis is a non-inflammatory form of cell death that occurs in a controlled manner, allowing for the orderly removal of cells without eliciting an immune response. In contrast, pyroptosis is often triggered by infections and results in cell lysis, releasing pro-inflammatory cytokines like IL-1β and IL-18, which promote inflammation (Choudhury et al., 2024). Other genes like CASP8, PARP1, CAPS9, and CYCS indirectly affect cell apoptosis and also interfere with other pathways.

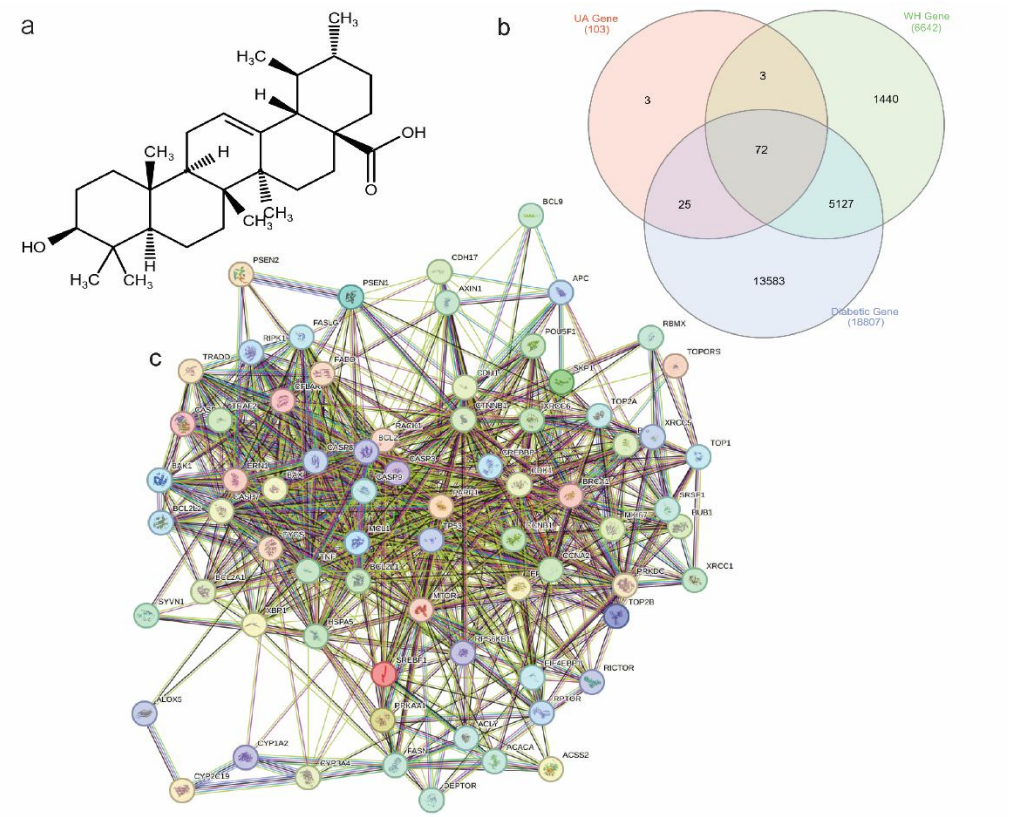

Figure 1. Ursolic acid's top target genes and proteins related to diabetic wound healing. a. Ursolic acid's structure, b. Venn diagram of ursolic acid and diabetic wound healing interfered genes, c. Protein-protein interaction (PPI) network of the intersecting genes.

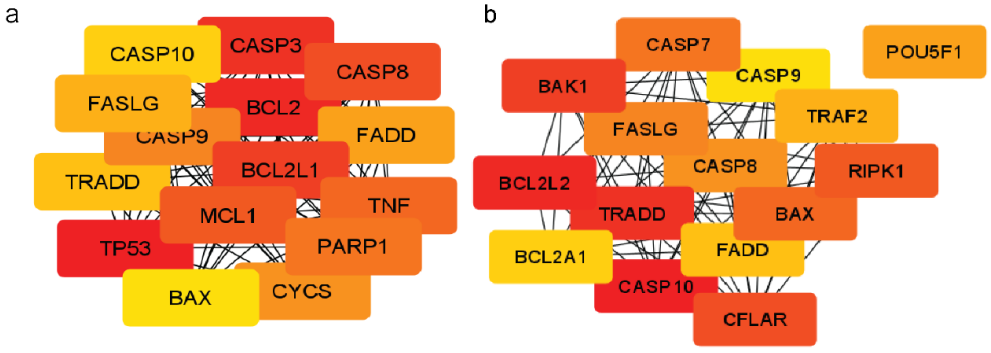

Figure 2. The clustering of the top 15 genes related to diabetic and wound healing according to (a) MCC and (b) DMNC algorithm in CytoHubba. Red to yellow represents a gradient of significance or ranking based on topological scores that indicate high to low hub genes, suggesting significant to less connectivity and biological importance. TP53: Tumor protein p53, BCL2: B-cell lymphoma 2, CASP3: Caspase-3, BCL2L1: B-cell lymphoma 2 like 1, CASP8: Caspase-8, MCL1: Myeloid cell leukemia-1, TNF: Tumor Necrosis Factor, PARP1: Poly (ADP-ribose) polymerase 1, CASP9: Caspase-9, CYCS: Cytochrome C, Somatic, FADD: Fas-associated protein with death domain, FASLG: Fas Ligand Gene, TRADD: TNFR1-associated death domain protein, CASP10: Caspase-10, BAX: Bcl-2-associated X protein, BCL2L2: Bcl-2-like protein 2, BAK1: BRI1-associated kinase 1, CFLAR: c-FLIP (FLICE-like inhibitory protein), RIPK1: Receptor-Interacting Serine/Threonine-Protein Kinase 1, CASP7: Caspase-7, POU5F1: POU domain, class 5, transcription factor 1, TRAF2: TNFR-associated factor 2, BCL2A1: B-cell lymphoma 2-related protein.

Table 1. Top 10 diabetic wound healing genes affected by ursolic acid based on the Maximal Clique Centrality (MCC) algorithm and their roles in diabetic wound healing

Gene/ Protein	Full Name	Biological function related to diabetic wound healing	References
TP53	Tumor protein p53	TP53, regulated by the PARP-1 transcription factor, is activated in response to oxidative stress induced by elevated glucose levels. This activation impact Superoxide Dismutase (SOD) expression, thereby affecting collagen secretion and promoting cell apoptosis.	(Setyawati et al., 2021; Tombulturk et al., 2023)
BCL2	B-cell lymphoma 2	BCL2, produced by the NF-ϰB transcription factor, exhibits anti-apoptotic and proliferative effects on cells.	(Ambrozova et al., 2017; Arya, 2014)
CASP3	Caspase-3	Caspase-3, induced by stress oxidative, Caspase-8, and Caspase-9. It is a key effector of caspase that triggers apoptotic cell death.	(Li et al., 2023; Boland et al., 2013; Mu et al., 2022)
BCL2L1	B-cell lymphoma 2 like 1	BCL2L1, the BCL2 family of proteins produced by NF-ϰB transcription factor. It is an anti-apoptotic and proliferative cell.	(Ambrozova et al., 2017)
CASP8	Caspase-8	Caspase-8, activated by TNF and TAK1 (Transforming growth factor-β-activated kinase 1). It activated Caspase-3 leading to cell death.	(Boland et al., 2013; Mu et al., 2022)
MCL1	Myeloid cell leukemia-1	MCL-1 is an antiapoptotic member of the BCL-2 protein family that protects cells against apoptosis.	(Meyerovich et al., 2017; Arya, 2014)
TNF	Tumor Necrosis Factor	TNF, regulated by NF-ϰB transcription factor. High glucose levels increase TNF activation. TNF activated Caspase-8 and inhibited activation of MCL1 which leads to cell death. A high level of TNF inhibits the migration of keratinocyte and endothelial cells and leads to apoptosis of that cell. TNF increases proinflammatory M1 macrophages and decreases anti-inflammatory M2 macrophages that induce inflammation.	(Ambrozova et al., 2017; Xu et al., 2013)
PARP1	Poly (ADP-ribose) polymerase 1	PARP-1 is a transcription factors of TP53 that influence the production of collagen. PARP-1, as an activator of NF-ϰB attenuates the production of various proinflammatory mediators.	(Banerjee et al., 2019; Li et al., 2023)
CASP9	Caspase-9	Caspase-9, produced by Procaspase-9 that induced by apoptosome. It activates Caspase-3 which leads to cell death.	(Boland et al., 2013)
CYCS	Cytochrome C, Somatic	CYCS binds to its receptor APAF1 which produces Caspase 9 that leads to cell apoptosis. CYCS in mitochondria inhibit the ATP and inhibit wound healing activation.	(Hunt et al., 2023; Xu et al., 2022).

Molecular docking

TP53 and TNF α are the proteins targeted by our molecular docking study. The goal of molecular docking is to provide a prediction of the interaction of a ligand-receptor complex, finding likely binding modes and binding energy using computational methods (Meng et al, 2012). The docking method was considered valid as it showed RMSD values 1.47 Å (TP53) and 1.068 Å (TNF α). Repetitions performed on the docking results showed consistent values. UA showed a binding energy value of -6.70 kCal/mol with TP53, while UA showed a lower binding energy value of -8.33 kCal/mol with TNF α. H-Bond occurred between UA and TP53 with two residues Tyr1552 and Met1584, and with Leu120 on TNF α. UA and TP53 showed Van Der Walls interaction with Trp1495, Asn1498, Tyr1502, Asp1521, Phe1553, Ala1585, Ile1587, Val1586 and hydrophobic interactions with Tyr1523, Leu1547, Phe1553, Ile1587 (Figure 3a, 3b). In contrast, TNF α exhibited van der Walls bonds with Leu57, Ser60, Gln61, Tyr119, Leu120, Gly121 and hydrophobic

interactions with Tyr151, Tyr59, and Tyr119 (Figure 3c, 3d, Table 2). These interactions were evaluated using geometric fingerprint interaction (IFP) rules by examining the distance and angle of the atom flags (Marcou & Rognan, 2007). This indicates a significant binding potential between UA and these two proteins.

DISCUSSION

Ursolic acid (UA) is a naturally occurring triterpene compound present in a variety of fruits and vegetables. It is extracted from the leaves of several plants such as rosemary, marjoram, lavender, thyme, and organum, as well as from fruit peels (such as apples), flowers, and berries. UA has garnered increasing attention due to its numerous beneficial properties, including anti-inflammatory, antioxidant, anti-apoptotic, and anti-carcinogenic effects across different tissues and organs (Seo et al., 2018). Given its widespread presence in plants and diverse health benefits, this study aims to explore the molecular mechanisms and pathways associated with UA through bioinformatics analysis. The PPI network facilitates gaining the top rank of MCC and DMNC algorithms. The MCC algorithm provides a more comprehensive analysis compared to other algorithms, displaying more essential proteins in both high and low-ranking protein lists (Chin et al., 2014; Lin et al., 2008). On the other hand, the DMNC algorithm provides neighborhood component-based analysis. Consequently, the MCC data provide a fundamental for predicting the top 10 genes and the molecular pathway of diabetic wound healing by UA, drawing on existing literature.

The process of wound healing is intricate and involves four primary stages: hemostasis, inflammation, new tissue formation, and remodeling (Xue et al., 2015). Our investigation elucidated the molecular mechanisms of UA within the diabetic wound healing pathway, specifically during the phases of inflammation and new tissue formation (Figure 4). Based on the results of bioinformatic testing, several genes influenced by UA play a role in cell apoptosis. Nevertheless, some genes also possess a regulatory function to impede apoptosis. The most prominent downregulated gene by UA in this study is TNF due to its role in various pathways. Its transcription is induced by the NF-κB transcription factor, and its cellular level is triggered by elevated blood sugar levels. TNF exerts a significant influence on inducing cell apoptosis, particularly in keratinocytes and endothelial cells, while concurrently impeding the migratory capabilities of these cells (Ambrozova et al., 2017; Xu et al., 2013). Furthermore, an excessive presence of TNF stimulates the activation of caspase-8, subsequently initiating caspase-3 and instigating cell apoptosis, while concurrently inhibiting MCL1, thereby amplifying cellular apoptosis (Boland et al., 2013; Mu et al., 2022). Additionally, TNF participates in the generation of M1 macrophages and suppresses M2 macrophages, which contribute to the inflammatory process (Ambrozova et al., 2017). Consequently, the TNF gene emerges as a potential target for impeding the diabetic wound healing facilitated by UA.

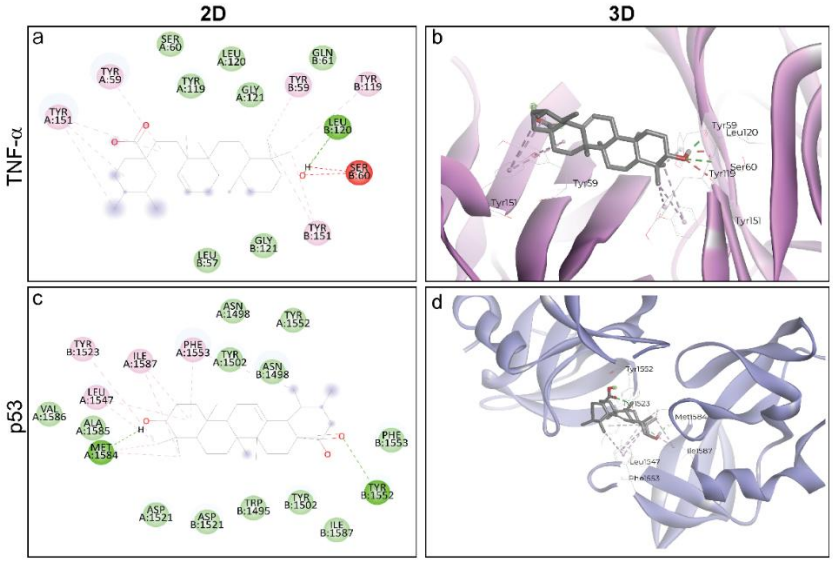

Figure 3. The binding poses of ursolic acid at selected protein in 2D view (a, c), and 3D view (b, d) at TNF-α (a, b) and P53 (c, d) binding pocket. Black, red, blue, and white indicate carbon, oxygen, nitrogen, and hydrogen atoms, respectively (b, d). Green, pink, orange, and purple represent Van der Walls, alkyl/pi-alkyl, pi-sulfur, and pi-sigma, respectively (a, c).

Table 2. Molecular docking results of ursolic acid's target protein

Protein	Binding energy kCal/mol	H-Bond residue	Hydrophobic residue	Van Der Walls residue
TP53 (8SWJ)	-6.70	Tyr1552, Met1584	Tyr1523, Leu1547, Phe1553, Ile1587	Trp1495, Asn 1498, Tyr1502, Asp1521, Phe1553, Ala1585, Ile1587, Val1586
TNF α (2AZ5)	-8.33	Leu120	Tyr151, Tyr59, Tyr119	Leu57, Ser60, Gln61, Tyr119, Leu120, Gly121

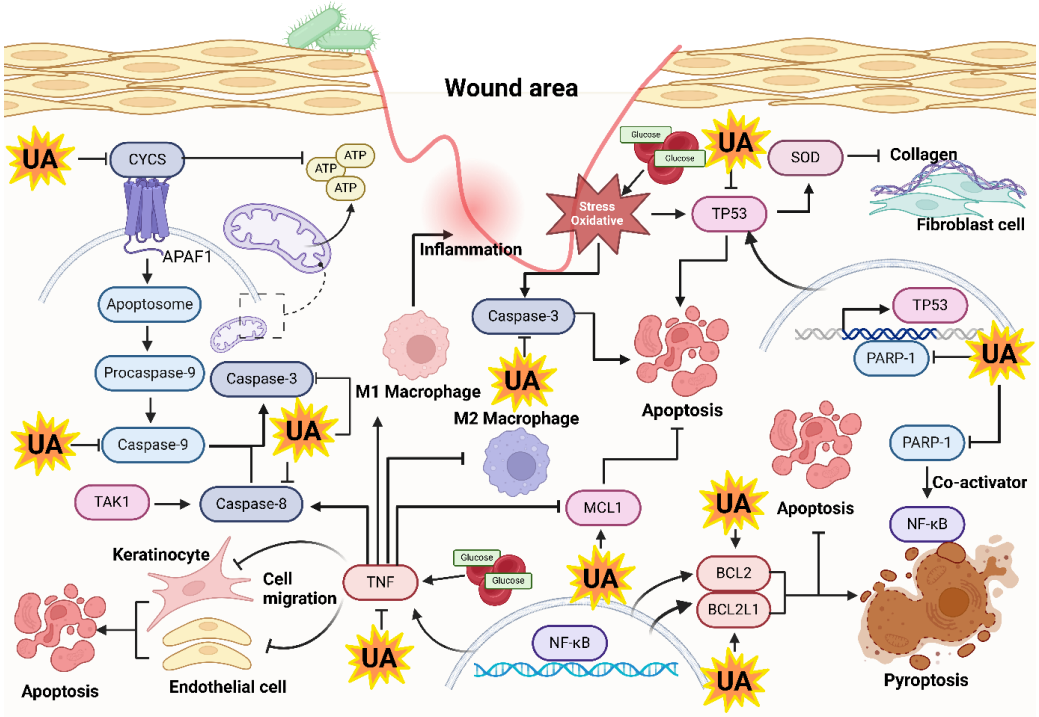

Figure 4. A predicted molecular cascade of ursolic acid (UA) in diabetic and wound healing of the skin. UA: Ursolic acid, TP53: Tumor protein p53, BCL2: B-cell lymphoma 2, CASP3: Caspase-3, BCL2L1: B-cell lymphoma 2 like 1, CASP8: Caspase-8, MCL1: Myeloid cell leukemia-1, TNF: Tumor Necrosis Factor, PARP1: Poly (ADP-ribose) polymerase 1, CASP9: Caspase-9, CYCS: Cytochrome C, Somatic, ATP: Adenosine Triphosphate, APAF1: Apoptotic Protease Activating Factor 1, TAK1: Transforming growth factor-β-activated kinase 1, NF-ϰB: Nuclear factor kappa-light-chain-enhancer of activated B cells, SOD: Superoxide Dismutase. The figure is prepared using Biorender.

UA additionally suppresses other genes directly associated with cellular apoptosis, specifically TP53 and Caspase-3, induced by oxidative stress. TP53, with the highest score in this bioinformatic analysis, impedes the production of collagen, thereby obstructing the wound-healing process (Setyawati et al., 2021; Tombulturk et al., 2023). The highest-ranked hub genes suggest they have crucial connectivity and potential biological importance in the wound-healing process. Conversely, the Caspase-3 gene forms a distinct pathway cluster with other caspase genes. Caspase-3 is activated by caspase 8 and caspase 9 (Li et al., 2023; Boland et al., 2013; Mu et al., 2022). Caspase-8 is primarily associated with the extrinsic apoptotic pathway through death receptor-mediated, while caspase-9 activates the intrinsic apoptotic pathway. Both caspase-8 and 9 activate caspase-3 mediates apoptosis during tissue remodeling to increase proliferation in wounds (Mcllwain et al., 2013). Caspase-8 is triggered by the TAK1 gene, while the Caspase-9 gene is generated from procaspase-9 resulting from the interaction between CYCS and its receptor, APAF1 (Boland et al., 2013; Mu et al., 2022; Hunt et al., 2023; Xu et al., 2022). In addition to producing Caspase-9, CYCS inhibits wound healing by inhibiting ATP production

in the cytoplasm (Hunt et al., 2023; Xu et al., 2022). Alongside genes that induce apoptosis, some genes inhibit it, namely BCL2, BCL2L1, and MCL1. Both BCL2 and BCL2L1 genes also play a role in cell pyroptosis (Ambrozova et al., 2017; Arya, 2014; Meyerovich et al., 2017). Therefore, UA induces all three genes contributing to the initiation phase of wound healing.

Since TNF-α interferes with various pathways and P53 possesses the highest bioinformatics score, we conducted molecular docking analysis of UA against both proteins. UA exhibits inhibitory potential against TNF-α (Figure 3a and Figure 3b) and P53 (Figure 3c and Figure 3d). The ribbon diagram illustrates the molecular interaction of UA within the active site pocket of TNF- and P53. Both docking configurations against TNF-α and P53 had Root Mean Square Deviation (RMSD) values of 1.068 and 1.47 Å, which are considered valid due to the values being under 2.00 Å (Meng et al., 2011). UA may inhibit TNF-α and P53 by binding to the protein's active site, indicating potential for further investigation as a diabetic wound healing.

CONCLUSION

To sum up, UA is predicted to orchestrate molecular cascades involving apoptosis and pyroptosis-related genes in various cell types such as fibroblasts, keratinocytes, endothelial, and erythrocytes. This study may support *in vitro* and *in vivo* studies of UA and or UA-containing natural products in diabetic wound healing activities. Accordingly, we expect this study will promote the development of UA-containing natural products as wound healing agents for diabetic patients.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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