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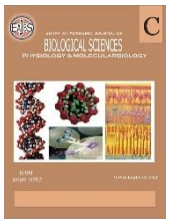

Assessment of Wound Healing Potential of *Punica granatum* Peel Extract in Rheumatoid Arthritis Mice: *In silico and In Vivo* Study

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ABSTRACT

Rheumatoid arthritis (RA) is the most widespread chronic autoimmune disorder worldwide, which is known to hinder wound healing. Therefore, this study aimed to investigate the effectiveness of pomegranate peel extract (PPEx) in accelerating wound healing in rheumatoid arthritis mice model through both in silico and in vivo approaches. Gas chromatography-mass spectrometry (GC-MS) analysis identified 31 active compounds in PPEx. The *in-silico* analysis explored the interaction between PPEx active compounds and inflammatory proteins (TNF-α, IL-6, NF-κB p65, RORγt, and T-bet) using the molecular docking method. Seven active compounds out of 31 had higher binding affinity, four of them (Lovastatin, ellagitannins, punicalagin, and ellagic acid) interact with all the five gene products under investigation. In the in vivo study, rheumatoid arthritis (RA) was induced in female Black 6 mice, and different doses of pomegranate peel extract (PPEx) (100 mg/kg and 200 mg/kg) were tested to assess their effects on healing. The evaluation of PPEx on wound healing, using the flow cytometry Annexin V test, indicated that the 200 mg/kg PPEx treatment could regulate the balance between early and late apoptosis and tissue proliferation. Furthermore, PPEx significantly accelerated wound closure rates and reduced the expression of inflammatory markers (TNF-α and IL-6) as well as transcription factors (NF-κB p65, RORγt, and T-bet), and the degree of reduction increased significantly with higher concentrations of PPEx. In conclusion, our findings suggest that PPEx has the potential as a natural therapeutic agent to enhance wound healing in RA patients by reducing inflammation and promoting balanced apoptosis.

INTRODUCTION

Rheumatoid arthritis (RA) is the most widespread and influential chronic autoimmune rheumatic disorder worldwide. Globally, RA affects around 1% of the global population (Guo *et al.*, 2018) It is more frequent in women and the elderly. RA is a systemic disorder that is considered to have both hereditary and environmental components.

It causes joint inflammation as well as bone degradation. Patients may experience various complications, including vasculitis, ulceration, delayed wound healing, deformities of the hands and feet, neuropathy, restrictions in daily activities, and a diminished quality of life. While medication can enhance the functional status of patients, it may also lead to delayed wound healing associated with disease-modifying antirheumatic drugs, a heightened risk of skin tears linked to corticosteroids, and an increased likelihood of infections due to biological therapies (Okita *et al.*, (2021).

Chronic wounds represent a significant discomfort that affects individuals globally (Muniandy *et al.*, 2018). The healing of wounds is a multifaceted process that involves four interrelated and sequential stages: hemostasis, inflammation, proliferation, and tissue remodeling (Guo and DiPietro, 2010). In healthy skin, the wound healing process initiates with the constriction of blood vessels and the creation of a fibrin clot (Huang *et al.*, 2000). During the inflammatory phase, cytokines released upon the activation of coagulation cascades attract white blood cells (WBCs) to the wound site, serving to safeguard the area from potential infections (Xue and Jackson, 2015). In the proliferative phase, fibroblasts become the predominant cells at the wound site (Diegelmann and Evans, 2004). These cells play a crucial role in the degradation of the fibrin clot and the synthesis of new extracellular matrix (ECM) components, including collagens (Poor *et al.*, 2022).

Inflammation is a complex defense mechanism of the body, where leucocytes migrate to damaged tissues from the vasculature, in order to destroy the agents that could cause tissue injury Gabay (2006). Acute inflammation is a response to infection agents such as pathogens (bacteria, fungi, viruses), effect of chemical, radiation, external injuries, wounds, and damage through foreign objects. Inflammation plays an important role in healing, but chronic inflammation can create several diseases and physical disfunctions (Stefanou *et al.*, 2020, Valentina *et al.*, 2020)).

Cytokines and transcription factors play critical roles in the inflammatory response. Interlukin-6 (IL-6) is a proinflammatory cytokine that stimulates the recruitment and activation of inflammatory cells including neutrophils and macrophages in the early phases of wound healing. Elevated levels of IL-6 have been seen in RA patients, while it has been linked to poor tissue healing and delayed wound closure. Moreover, Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that has a significant role in the development of RA. It can severely influence wound healing by inducing apoptosis, decreasing cell proliferation, and hindering angiogenesis (Jang *et al.*, 2021).

The transcription factor NF-κB p65 (nuclear factor-kappa B p65) regulates inflammatory gene expression and has been linked to the development of RA and delayed wound healing (Liu *et al.*, 2017). Activating NF-κB p65 increases the production of pro-inflammatory mediators such as cytokines, chemokines, and adhesion molecules, which can prolong the inflammatory response and hinder wound healing (Giridharan and Srinivasa, 2018).

Moreover, the transcription factors RORγt (retinoic acid-related orphan receptor gamma) and T-bet (T-box produced by T cells) play crucial functions in the proliferation and function of inflammatory T cell subsets, such as Th17 and Th1 cells, which contribute to the chronic inflammatory environment in RA [12-13]. RORγt regulates Th17 cells, which produce IL-17 and cause joint damage and systemic inflammation in RA (Mickael *et al.*, 2020). Whereas T-bet regulates Th1 cells, which generate IFN-γ and contribute to RA by activating macrophages and inducing pro-inflammatory cytokines (Anderson, *et al.*, 2010).

Many herbal components that are supposed to treat and repair wounds quickly are being studied. Pomegranate (Punica granatum) has long been utilized in traditional

medicine practices to address a wide range of ailments and pathological situations. Because of its positive health effects, it has become the subject of contemporary study (Baradaran *et al.,* 2020). The pomegranate's peel, seeds, and arils all contain physiologically active chemicals. The pomegranate peel, which makes up 26-30% of the fruit's total weight (Ismail *et al.,* 2012), is regarded as the most medicinal component. It contains various minerals, essential amino acids, sugars, unsaturated and saturated fatty acids, tocopherols, sterols, terpenoids, and high levels of polyphenols such as anthocyanins, flavonoids, ellagitannins, and alkaloids (Čolic *et al.*, 2022, Baradaran *et al.,* 2020, and Omer *et al.,* 2019).

Molecular docking is a computer approach employed to forecast the interaction between two molecules, creating a binding model. In many drug discovery applications, the process of docking in which a tiny molecule is matched with a larger macromolecule is called protein-ligand docking. The essence of ligand-protein docking is to predict the predominant binding model ligand with a protein of three-dimensional structure. In recent times, docking has also been utilized to forecast how two macromolecules, such as proteins, bond together. This is known as protein-protein docking. This approach has demonstrated its use in medicinal chemistry and drug development by offering a detailed understanding of molecular recognition. Moreover, it saves time and effort (Dawen *et al.*, 2024, Prieto-Martinez *et al.*, 2018).

Therefore, the objective of this research was to examine the impact of pomegranate peel extract (PPEx) on the process of wound healing in a rheumatoid arthritis mouse model and try to understand its role in wound healing and correlate between the *In Silco* and *in vivo* study. The results of this study might give significant knowledge on the potential of PPEx as a therapeutic addition to improve wound healing in people with rheumatoid arthritis. This could provide a natural and potentially cost-effective alternative to conventional wound care methods.

MATERIALS AND METHODS

1. Analysis of PPEx Using GC-MS Device:

The active compounds of PPEx were assessed using the Agilent 5977A gas chromatograph mass spectrometry system, which is integrated with the GC Clarus 500 Perkin Elmer system. This system features the AutoSampler [AOC-20i + s] for the analysis of various compounds and utilizes bound gas chromatography. The chromatograms produced by this equipment are automatically programmed into the machine.

2. Molecular Docking:

2.1. Preparation of Protein/ Receptor: Compounds authenticated in the PPEx were exploited using virtual in-silico studies, first docking of the compounds upon five essential gene products namely TNF-α, IL-6, NF-kB p65, RORϒt, and T-Bet following the steps below:

- From the database of PubChem (https://pubchem.ncbi.nlm.nih.gov/) authenticated compounds in the GC-MS of the ethanolic extract were downloaded in 3D.sdf format, compounds were subjected to PYRX software as a small library, and energy minimization was performed.
- Afterward, protein preparation was achieved using PDB entries of five potential targets TNF-α (PDB: 4E4S), IL-6 (PDB: 8QY5) NF-KB P65 (PDB: 9bdv) RORϒt (PDB: 7LUK) and T-Bet (PDB: 4BUH). Docking was performed between ligand and protein molecules using PyRx having AutoDock vina Plugin (Kondapuram *et al*., 2021).

2.2. Grid Dimensions: The grid box dimension values in angstrom for 4E4S receptor were kept at X: 55.4541, Y: 58.8621, and Z: 59.1911. Whereas The grid box dimensions (Å) size X=83.1523; size Y=83.2036; size Z=62.8114 for 8QY5, The grid box dimensions (Å) size X=88.3299; size Y=92.4211; size Z=69.9581 for 9bdv The grid box dimensions (Å) size X=56.4105; size Y=53.6384; size Z=42.7068 for 7LUK finally; the dimension values (Å) for 4BUH entry were X:41.2205, Y: 56.0790, and Z: 58.7571.

By setting the exhaustiveness value to eight, a series of dockings were conducted to obtain nine unique poses of the ligand molecule. Upon concluding the docking procedure, the pose that exhibited the lowest binding energy was chosen for the purpose of visualizing the interaction between the ligand and the protein through Discovery Studio 2022 [https://10.0.142.116/pharmaceutical-sciences.543].

2.3. ADMET Analysis: In order to evaluate the drug-like characteristics of the natural compounds that exhibited the greatest binding affinity scores by molecular docking with TNF-α, IL-6, NF-kB P65, RoRϒT, and T-bet proteins, ADMET STAR (https:// Immd. ecust.edu.cn/admetstar2/) was utilized.

ADMET analysis is crucial in the development of drugs and environmental risk assessment as it accurately characterizes the features of medicinal compounds and environmental contaminants. The most important pharmacokinetic parameters assessed by ADMET evaluation consist of the blood-brain barrier, CYP4502C9 activity, intestinal absorption in human, and Caco-2 permeability (Cheng *et al.,* 2012). Lipinski's rule of five parameters (RO5) states that particles must have a molecular weight below 500 g/mol, a topological polar surface area (TPSA) below 140 × 2, an AlogP value below 5, number of hydrogen bond donors and acceptors below 5 and10, respectively (Yang *et al.,* 2019; Daina *et al.,* 2017). Furthermore, the hepatotoxicity of the substances was assessed using this approach.

3. Pomegranate Peel Extraction (PPEx):

The pomegranate peel was carefully peeled from the fruit, dried for 4-6 days at room temperature, then milled into powder. 100 g sample of powdered pomegranate peel was extracted with 70% ethanol, using a solid to solvent ratio of 1:10. The extraction process was conducted in an ultrasonic bath at a temperature of 60°C for a duration of 40 minutes. Following filtering, the extract underwent evaporation using a Büchi R-210 rotary evaporator from Flawil, Switzerland. The resulting raw material was subsequently utilized for further extraction and characterization.

4. Animal Experiment Design:

Twenty-five adolescent Black 6 female mice, aged 7-8 weeks and weighing 25-30 g, were obtained from the animal care section at the National Research Centre in Egypt. Mice were distributed at random into five groups, each consisting of six mice: group 1, non-Rheumatoid arthritis and unwounded mice (normal control; N); group 2, non-Rheumatoid arthritis mice with wounds as a negative control (NW); group 3, rheumatoid arthritis mice with wounds as a positive control(RW); group 4, rheumatoid arthritis mice with wounds treated with 100 mg/kg PPEx (RWPPEx100) for 14 days; group 5, rheumatoid arthritis mice with wounds treated with 200 mg/kg PPEx (RWPPEx200) for 14 days. The dimensions of the wound were documented at intervals of 3 days during the experiment. Following a 24-hour period after the final dosage, heparinized whole blood was obtained by puncturing the retro-orbital plexus under mild ether anesthesia. All mice were then sacrificed by cervical dislocation to collect the wound tissue for laboratory testing.

4.1. Induction of Adjuvant-Induced Arthritis: The RA model was established using Black 6 female mice around 7-8 weeks old. Following general anesthesia, one hundred µL of vortexed CFA was injected using 1 mL insulin syringes. The injection process involved depositing 20µL into the ankle joint cavity of the left hind foot, followed by injecting the remaining 80µL in four doses into the tissues surrounding the joint. Three days post-injection, the mouse's left hind ankle joint was graded. The joint diameter was assessed using a pocket thickness meter at intervals of 3 days.

4.2. Wound Model and Tissue Preparation: The excision wound model was used to monitor wound healing in normal and Rheumatoid arthritis mice. The mice underwent anesthesia through an intraperitoneal injection of chloral hydrate at a dosage of 400 mg/kg. Following the shaving of the dorsal area of each mouse, two full-thickness excisional wounds, each measuring

5 mm in diameter, were created using a standard biopsy punch. After 14 days of the experiment, the mice were anesthetized using light ether anesthesia, and whole blood was drawn from the retro-orbital plexus into heparinized tubes before the mice were sacrificed. The wound and the surrounding tissue were removed from the pelt. Wounds were either frozen in liquid nitrogen for RNA and protein analysis or fixed in 10% formalin for histological analysis. Concurrently, the treatment groups received 100 and 200 mg/kg/d of PPEx via intragastric administration, commencing next days after the wounding and continuing for 14 consecutive days. The control mice (N) and NW model mice were administered a solution of normal saline.

5. Analysis of Apoptosis by Flow Cytometry (FCM):

Skin cells untreated and treated with PPEx were harvested and collected into 15 mL falcon tubes. The cells were re-suspended in a complete growth medium to 4×10^5 cells/mL followed by adding Annexin V-FITC to detect apoptosis following the manufacturer's (eBioscences in Vienna, Austria) protocols. The samples were immediately analyzed using a flow cytometer. The analysis was conducted by triplicate determination on a minimum of three distinct experiments.

6. RNA Extraction and cDNA Synthesis:

Total RNA from the skin tissues of each mouse was isolated using TRIzol (Invitrogen), as per the manufacturer's instructions, and quantified by spectrophotometer. The RNA quality was determined from the 260/280 ratio. The cDNA synthesis was performed using high-capacity cDNA reverse transcription kit (Applied BioSystems), in accordance with the manufacturer's instructions.

6.1. Quantification of mRNA expressions in skin tissue using RT-PCR:RT-PCR analysis was performed by using the above cDNA and gene-specific primers in an ABI Prism 7500 System (Applied BioSystems). The reaction mixture consists of 1.3 µl of the cDNA sample, forward and reverse primer (10 µM each), 12.5 µ1 of SYBR Green Universal Master Mix, and 11 µ1 of nuclease-free water. The reaction conditions were: 95℃ for 2 min and 40 cycles of 95℃ for 15s, the annealing temperature was at 61°C for TNF-α, 65°C for IL-6, and 60°C for NF-kB p65, RORϒt, T-Bet and β-actin and 72℃ for 20s. With the β-actin gene as the reference, relative gene expression was determined by $2^{-\triangle\triangle Ct}$ method. The primers used for the mouse genes are listed in Table (1).

Table (1): Primer sequence for the genes used in qRT-PCR.

Gene	Primer sequence		Reference
B-actin	5'-CGTGCGTGACATCAAAGAGAA-3'	5'-TGGATGCCACAGGATTCCAT-3'	Yamakawa *et al.*, 2011
IL-6	5'-**GCCTCTTCTCATTCCTGCTTG**-3'	5'-**CTGATGAGAGGGAGGCCATT**-3'	Yamakawa *et al.*, 2011
TNF-α	5'-ACGGCCTTCCCTACTTCACA-3'	5'-CATTTCCACGATTTCCCAGA-3'	Yamakawa *et al.*, 2011
NF-kB p65	5′-AGGCAAGGAATAATGCTGTCCTG-3′	5′-AGGCAAGGAATAATGCTGTCCTG-3′	Le *et al.*, 2018
RORϒt	5'-TCACCTGTG AGGGGTGCAAG-3'	5'-GTTCGG TCAATGGG GCAGTT-3'	Yoh *et al.*, 2011
T-Bet	5'-ATGTTTGTGGATGTGGTCTTGGT-3'	5'-CGGTTCCCTGGCATGCT-3'	Pei *et al.*, 2019

7. Statistical Analysis:

The variation between the groups was evaluated using a one-way analysis of variance (ANOVA) using Statistical Product and Service Solutions (SPSS) Statistics version 23. Means were compared using Tukey's honestly significant difference test. The data was given as mean ± standard error (SEM), and the significance level was set at $p < 0.05$.

RESULTS

The ultimate goal of this research was to find out the importance of PPEx as an anti-inflammatory agent for healing wounds. Therefore, in this study, the effect of PPEx on the profile of cytokines (IL-6 and TNF-α) and

three transcription factor genes (RORϒt, NF-kB P65, and T-bet) expressed in the wounded rheumatoid and non-rheumatoid mice was investigated in silico and in vivo.

Identification of Active Compounds in PPEx:

Gas chromatography (GC) analysis of Pomegranate Peel Extract (PPEx) identified 31 active compounds. Among these, seven compounds demonstrated higher binding affinity to the receptors of five inflammatory mediators (TNF-α, IL-6, NF-κB P65, RORγt, and T-bet) compared to the control drugs (Sulfadiazine, Acetaminophen, and Ibuprofen) **(**Table 2**)**.

Molecular Docking Simulations for PPEX Active Compounds and The Five Inflammatory Mediators (TNF-α, IL-6, NF-κB P65, RORγt, and T-bet):

The binding affinities of the seven most potent compounds and control drugs are presented in Table (2). Four of them (Lovastatin, Ellagitannins, Punicalagin, and Ellagic acid) interact with all the five proteins. The obtained data showed that 6 compounds with a high score for TNF-α gene product were Lovastatin, Pirfenidone, Ellagitannins, Punicalagin, Punicalin, and ellagic acid with scores of (-7.5, -7.7, -9.3, -10.0, -8.3, and -8.9 kcal/mol, respectively). Whereas for IL-6 protein the compounds were Lovastatin, Ellagitannins, Punicalagin, Punicalin, and ellagic acid which were scored (-6.6, -8.3, -8.1, -7.7, and -6.9 kcal/mol, respectively). Meanwhile, the compounds for NF-κB P65 gene product were Lovastatin, Ellagitannins, Punicalagin, Punicalin, and, ellagic acid with scores of (-9.0, -11.5, -10.4, -10.1, and -9.4 kcal/mol, respectively), for RORγt protein, were Lovastatin, -Oleyl-alcohol, heptafluorobutyrate, Ellagitannins, Punicalagin, and ellagic acid (-9.5, -7.3, -9.7, -7.5, and -8.8 kcal/mol, respectively), and for T-Bet gene product, the compounds were Lovastatin, Ellagitannins, Punicalagin, and ellagic acid with scores of (-8.6, -10.8, -7.9, and -9.0 kcal/mol, respectively). The 2D molecular interaction of the TNF-α, IL-6, NF-KB P65, RORγt, and T-Bet proteins with the compounds that linked with their receptors was shown in Figures (1 to 5).

Table 2: Binding Affinity of Control Drugs and The Highest Active Compounds to Target Proteins (TNF-α, IL-6, NF-κB P65, RORγt, and T-bet).

No.	Ligand name	Chemical Class	Binding affinity				
			TNF-α	**IL-6**	**NF-κB P65**	**RORγt**	**T-bet**
Control	Sulfadiazine	Antibacterial	-6.4	-6.1	-7.6	-6.6	-7.2
	Acetaminophen	Phenols	-6.2	-5.3	-5.8	-5.6	-6.1
	Ibuprofen	Non-steroidal anti-inflammatory drug (NSAID)	-7.2	-5.7	-6.3	-7.0	-6.3
1	Lovastatin	fatty acid ester	-7.5	-6.6	-9.0	-9.5	-8.6
2	Pirfenidone	anti-inflammatory and antioxidant	-7.7	-5.9	-6.5	-6.9	-6.7
3	Oleyl-alcohol, heptafluorobutyrate	Fatty alcohol	-5.2	-4.6	-6.9	-7.3	-6.3
4	Ellagitannins	Polyphenols	-9.3	-8.3	-11.5	-9.7	-10.8
5	Punicalagin	Tannin	-10.0	-8.1	-10.4	-7.5	-7.9
6	Punicalin	Tannin	-8.3	-7.7	-10.1	-6.8	-7.2
7	Ellagic acid	Oxidative aromatic	-8.9	-6.9	-9.4	-8.8	-9.0

ADMET Properties:

The ADMET (absorption, distribution, metabolism, ecretion, and toxicity) profile of the top compounds revealed that Ellagitannins, Punicalagin, and Punicalin did not adhere to Lipinski's criteria, as their molecular weight was above 500 g/mol, TPSA was bigger than 140 (Å), and the number of hydrogen bond acceptors and donors exceeded 10 and 5, respectively

(Table 3). Moreover, the TPSA scores of Lovastatin, Pirfenidone, Oleyl-alcohol, heptafluorobutyrate, were within range whereas Ellagitannins, Punicalagin, Punicalin, and Ellagic acid exhibited higher TPSA values. In drug development, water solubility (logS) is a crucial metric, and all chosen compounds have values < 5.

Except for three compounds (Ellagitannins, Punicalagin, and Punicalin), the molar refractivity (MR) of the tested compounds fell within the acceptable range of 40-130. Skin permeability (log Kp) is an important statistic in transdermal drug research, with values larger than 2.5 indicating reduced skin permeability. Furthermore, Lovastatin, Pirfenidone, and Ellagic acid exhibited high bioavailability/GI absorption, while the remainder of the substances had lesser levels.

Lovastatin and Pirfenidone were discovered to pass the blood-brain barrier (BBB), but the remaining chemicals had limited distribution to the brain.

Cytochrome p450 (CYP450) inhibitors determine if a chemical will inhibit CYP450 and its isoforms.

It was found that Pirfenidone and Ellagic acid inhibit CYP1A2, Moreover, Lovastatin inhibits both CYP2C9 and CYP3A4. The rest of the compounds don't have any inhibition activity with all the CYP450 isoforms.

Table 3: ADMET Properties of The Highest Active Compounds.

ADMET	Lovastatin	Pirfenidone	Oleyl-alcohol, heptafluorobutyrate	Ellagitannins	Punicalagin	Punicalin	Ellagic acid
Molecular weight (g/mol)	404.54	185.22	464.50	636.47	1084.72	782.53	302.19
Number of H-bond acceptors	5	1	9	18	30	22	8
Number of H-bond donors	1	0	0	11	17	13	4
Water solubility (logS)	-3.19	-1.94	-8.00	-3.65	-4.73	-2.71	-2.94
TPSA (Å2)	72.83	22.00	26.30	310.66	518.76	385.24	141.34
Molar refractivity (MR)	113.92	57.01	109.15	142.86	250.86	180.45	75.31
Log Kp (skin permeation) cm/s	-5.74	-6.11	-1.69	-9.93	-11.67	-11.28	-7.36
Bioavailability score	0.55	0.55	0.55	0.17	0.17	0.17	0.55
GI absorption	High	High	Low	Low	Low	Low	High
Lipinski rule	Yes	Yes	Yes	No	No	No	Yes
Blood-Brain Barrier (BBB)	Yes	Yes	No	No	No	No	No
CYP1A2 inhibitor	No	Yes	No	No	No	No	Yes
CYP2C19 inhibitor	No	No	No	No	No	No	No
CYP2C9 inhibitor	Yes	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No	No
CYP3A4 inhibitor	Yes	No	No	No	No	No	No

Molecular Interactions

2D molecular interaction studies revealed specific binding patterns for each compound with the target proteins (Figs. 1-5). Table (4), illustrates the numerous types of interactions produced by proteins and compounds in this study. Key interactions included various types for each protein. TNF-α predominantly exhibited conventional hydrogen bonds, alkyl interactions, and pi-pi interactions. For IL-6, conventional hydrogen bonds, carbon hydrogen bonds, and pi-alkyl interactions were observed. Moreover, NF-κB P65 displayed a mix of a mix of conventional hydrogen bonds, alkyl interactions, and attractive charge interactions. Whereas RORγt showed prominent conventional hydrogen bonds, alkyl interactions, and pi-pi stacked interactions were prominent. T-bet: Conventional hydrogen bonds, alkyl interactions, and attractive charge

interactions. Table 4 presents the specific amino acid residues involved in the binding process for each protein-ligand pair. This detailed analysis provides insights into the molecular basis of the observed binding affinities and potential mechanisms of action for the PPEx compounds.

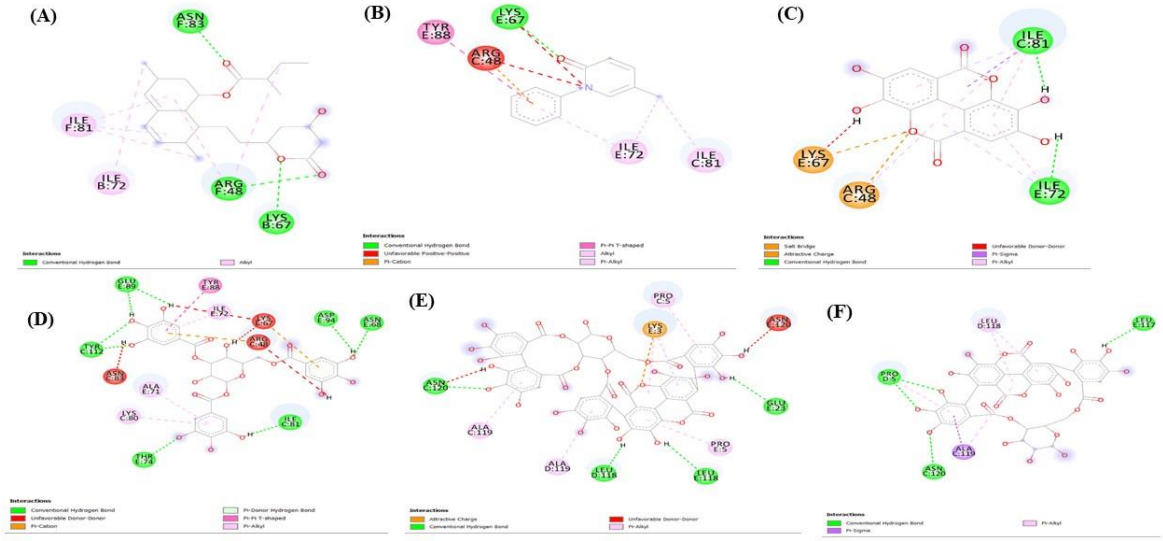

Fig. 1: 2D Molecular Interaction of TNF-α With (A) Lovastatin, (B) Pirfenidone, (C) Ellagic-acid, (D) Ellagitannins, (E) Punicalagin, and (F) Punicalin

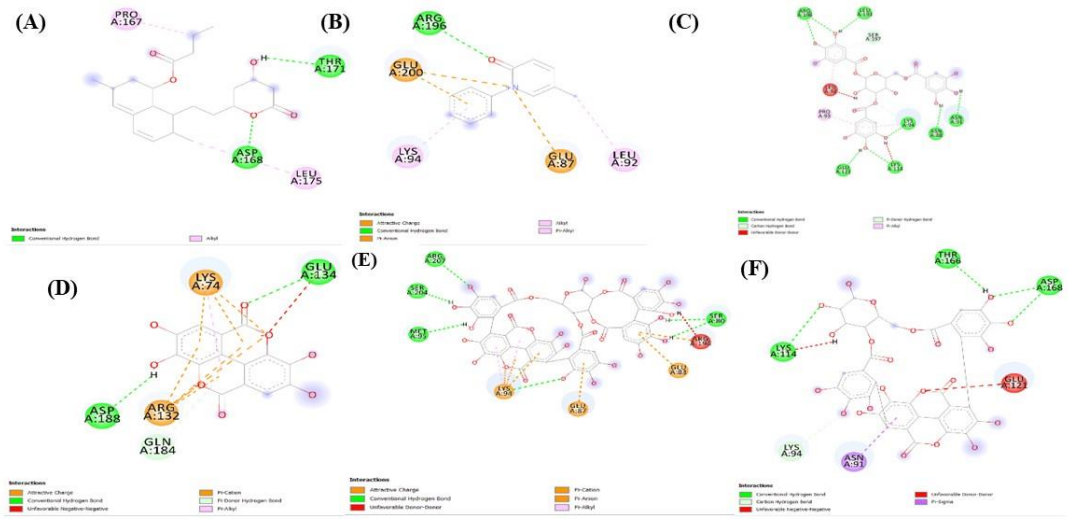

Fig. 2: 2D Molecular Interaction of IL-6 With (A) Lovastatin, (B) Pirfenidone, (C) Ellagitannins, (D) Ellagic-acid, (E) Punicalagin, and (F) Punicalin

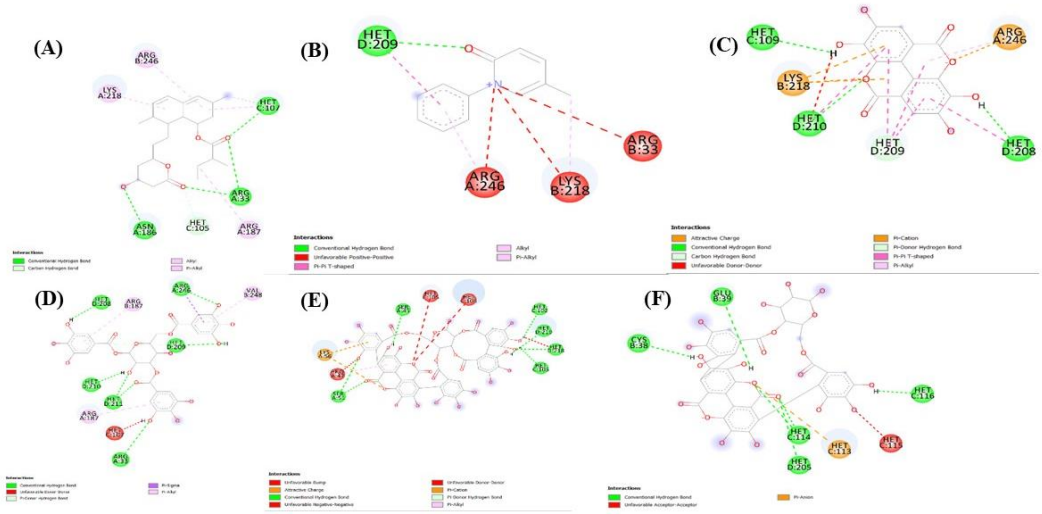

Fig. 3: 2D Molecular Interaction of NF-kB P65 With (A) Lovastatin, (B) Pirfenidone, (C) Ellagic-acid, (D) Ellagitannins, (E) Punicalagin, and (F) Punicalin

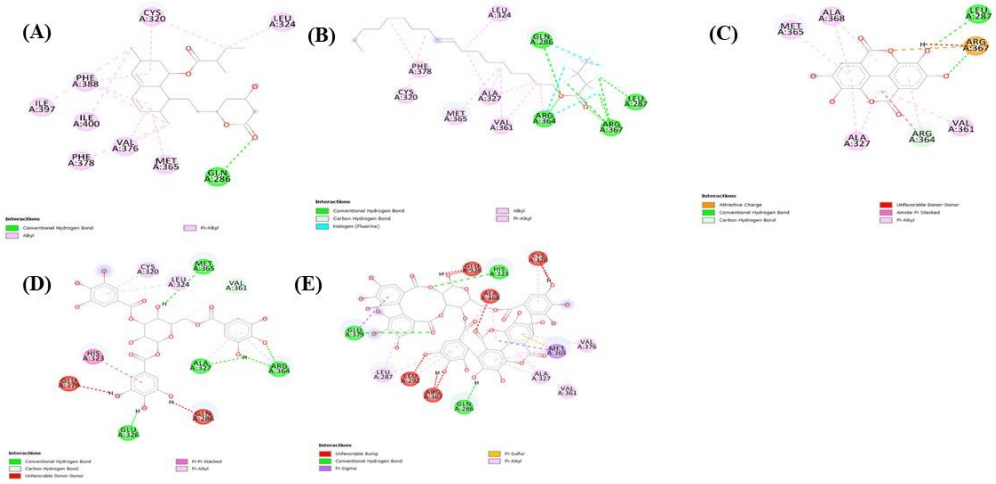

Fig. 4: 2D Molecular Interaction of RORγT With (A) Lovastatin, (B) Oleyl alcohol, heptafluorobutyrate, (C) Ellagic-acid, (D) Ellagitannins, and (E) Punicalagin.

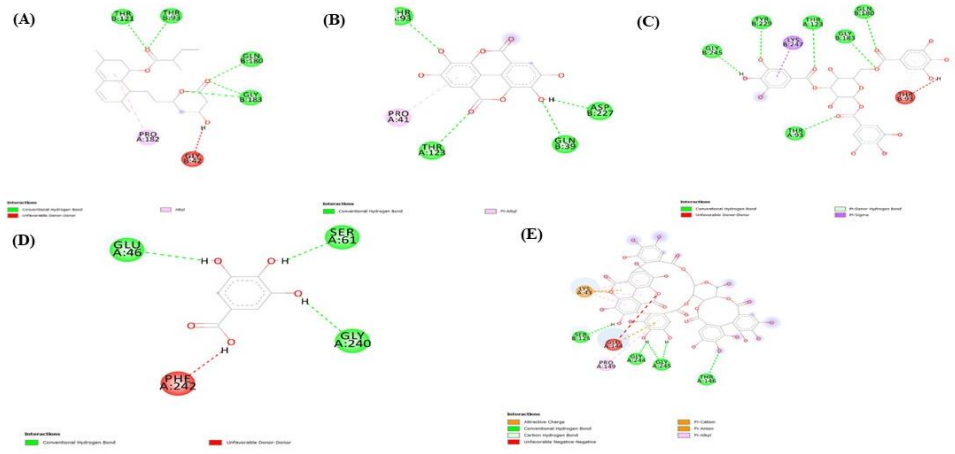

Fig. 5: 2D Molecular Interaction of T-Bet With (A) Lovastatin, (B) Ellagic-acid, (C) Ellagitannins, (F) Punicalagin, and (G) Punicalin

Table 4: Type of Interaction and Residue Position Between Docked TNF-A, IL-6, NF-κb P65, RORϒt, and T-Bet Proteins and Compounds.

Protein	Ligand	Interaction	Residue and Position
TNF-α	Lovastatin	Conventional	ASN (F:83), ARG (F:48), and LYS (B:67)
		Alkyl	ILE (F:81), and ILE (B:72)
	Pirfenidone	Conventional	LYS (E:67)
		Alkyl	ILE (E:72), and ILE (C:81)
		PI-PI T-shaped	TYR (E:88)
		Pi-Cation	ARG (C:48)
	Ellagitannins	Conventional	ASN E:68, ASP E:94, GLU E:89, TYR C112, THR E:74, AND ILE C:81
		PI- Alkyl	ILE (E:72), LYS C:80, ALA E:71
		Pi-Cation	LYS (E:67), and ARG (C:48)
		PI-PI T-shaped	TYR (E:88)
	Punicalagin	Conventional	ASN C:120, LEU D:118, LEU E: 118, and GLU E:23
		PI- Alkyl	Pro c:5, PRO E:5, ALA C:119, ALA D:119
		Attractive charge	LYS (E:3)
	Punicalin	Conventional	LEU E:117, PRO D:5, and ASN C:120
		PI- Alkyl	LEU D:118
		PI-Sigma	ALA C:119
	Ellagic acid	Conventional	ILE (C:81), and ILE (E:72)
		Salt Bridge / attractive charge	LYS (E:67), and ARG (C:48)
IL-6	Lovastatin	Conventional	THR A:171, ASP A:168
		Alkyl	LEU A:175, PRO A:167
	Ellagitannins	Carbon	SER A:197
		PI- Alkyl	PRO A:93
		Conventional	LEU A:193, ARG A:196, ASN A:91, ASN A:88, LYS A:94, LYS A:114, GLU A:121
	Punicalagin	Pi-Cation/ Attractive charge/ Pi-Anion	GLU A:83, GLU A:87, LYS A:94
		Conventional	SER A:80, ARG A:207, SER A:204, MET A:95
	Punicalin	Carbon	LYS A:94
		PI-Sigma	ASN A:91
		Conventional	ASP A:168, THR A:166, LYS A:114
	Ellagic acid	Attractive charge/ Pi-Cation	LYS A:74, ARG A:132
		Conventional	GLU A:134, ASP A:188
		PI-Donor	GLN A:184
NF-kB P65	Lovastatin	Alkyl / PI- Alkyl	ARG B:246, LYS A:218, and ARG A:187
		Conventional	HET C:107, ARG A:33, and ASN A:186
		Carbon	HET C:105
	Ellagitannins	Conventional	ARG A:246, ARG A:33, HET D:208, HET D:209, HET D:210, HET D:211
		Pi-Alkyl	ARG A:187, ARG B:187, and VAL B:248
	Punicalagin	Attractive charge/ Pi-Cation	LYS A:56
		Conventional	SER A:51, SER A:45, HET C:102, HET D:219, HET D:218, HET C:103
	Punicalin	Pi-Anion	HET C:113
		Conventional	HET C:116, GLU B:39, CYS B:38, HET C:114, HET D:205
	Ellagic acid	Carbon	HET D:209
		Conventional	HET D:208, HET D:210, and HET C:109
		Attractive charge / PI-Cation	ARG A:246, LYS B:218

RORϒT	Lovastatin	Conventional	GLN A:286
		Alkyl/Pi-Alkyl	LEU A:324, CYS A:320,PHE A:388,ILE A:397, ILE A:400, VAL A:376, PHE A:378, MET A:365
	Oleyl-alcohol, heptafluorobutyrate	Conventional	GLN A:286, ARG A:364, ARG A:367, LEU A:287
		Alkyl/Pi-Alkyl	LEU A:324, PHE A:378, CYS A:320, MET A:365, ALA A:327, VAL A:361
	Ellagitannins	Carbon	VAL A:361
		PI-PI Stacked	HIS A:323
		PI-Alkyl	CYS A:320, LEU A:324
		Conventional	GLU A:326, ALA A:327, ARG A:364,MET A:365
	Punicalagin	PI-Sigma	MET A:365
		Conventional	HIS A:323, GLU A:379, GLN A:286
		PI-Alkyl	VAL A:376, ALA A:327,VAL A:361, LEU A:287
	Ellagic acid	Carbon	ARG A:364
		Attractive charge	ARG A:367
		Conventional	LEU A:287
		PI-Alkyl	VAL A:361, ALA A:327, ALA A:368, MET A:365
T-Bet	Lovastatin	Alkyl	PRO A:182
		Conventional	GLY B:183, GLN B:180, THR B:93,THR B:121
	Ellagitannins	Pi-Sigma	LYS B:247
		Conventional	THR A:93, GLY B:245, TYR B: 229, THR A:123, GYL B:183, GLN B:180
	Punicalagin	PI-Alkyl	PRO A:149
		Conventional	THR A:146, GLY A:245, GLY A244, SER B:125
		Attractive charge/ Pi-Cation/Pi-Anion	LYS A:43
	Ellagic acid	PI-Alkyl	PRO A:41
		Conventional	THR A:93, THR A:123, GLN B:39, ASP B:227

Our docking results revealed that the anti-inflammatory docked ligand scores ranged from -4.6 to – 11.5. The present study revealed that 7 out of 31 active compounds had the higher docking scores and 3 of them interacted with all the 5 proteins under investigation. This may explain the way that PPEx effects on wound healing.

The *in silco* studies demonstrated that PPEx has considerable anti-inflammatory action and plays an important role in wound healing.

Wound Healing Rate in RA Mice:

The wound healing efficacy of PPEx was established by investigating the wound closure rate (Fig. 6). The results showed that the wound closure rate of the RWPPEx200 group was significantly decreased on days 4, 8, and 12 compared to NW and RW. Moreover, the treatment dose of 200 mg/kg is better than 100 mg/kg.

Effect of PPEx on Apoptosis:

To investigate the effect of PPEx in inducing apoptosis on wounds in RA patients, skin biopsy cells from mice were isolated and evaluated as described in Material and Methods. The effect of PPEx significantly reduced the early (11.5%, and 4.2%) and late (19.8%, and 5.51%) apoptosis after treating cells with 100, and 200 mg/ml, respectively than the NW (18.3%, and 11.4%, respectively) and RW (24.6%, and 14.5%, respectively). Meanwhile, the 200 mg/ml treatment (11.5%, 9.8%) induced a significant augmentation compared to the 100 mg/ml treatment (4.2%, and 5.51%) in both early and late apoptosis, respectively (Fig. 7 and Table 5).

The outcomes of this study will shed light on the potential of pomegranate extract in encouraging rapid wound healing via its effects on apoptosis. Understanding the processes behind pomegranate-mediated tissue restoration may help in the development of innovative wound healing therapeutics.

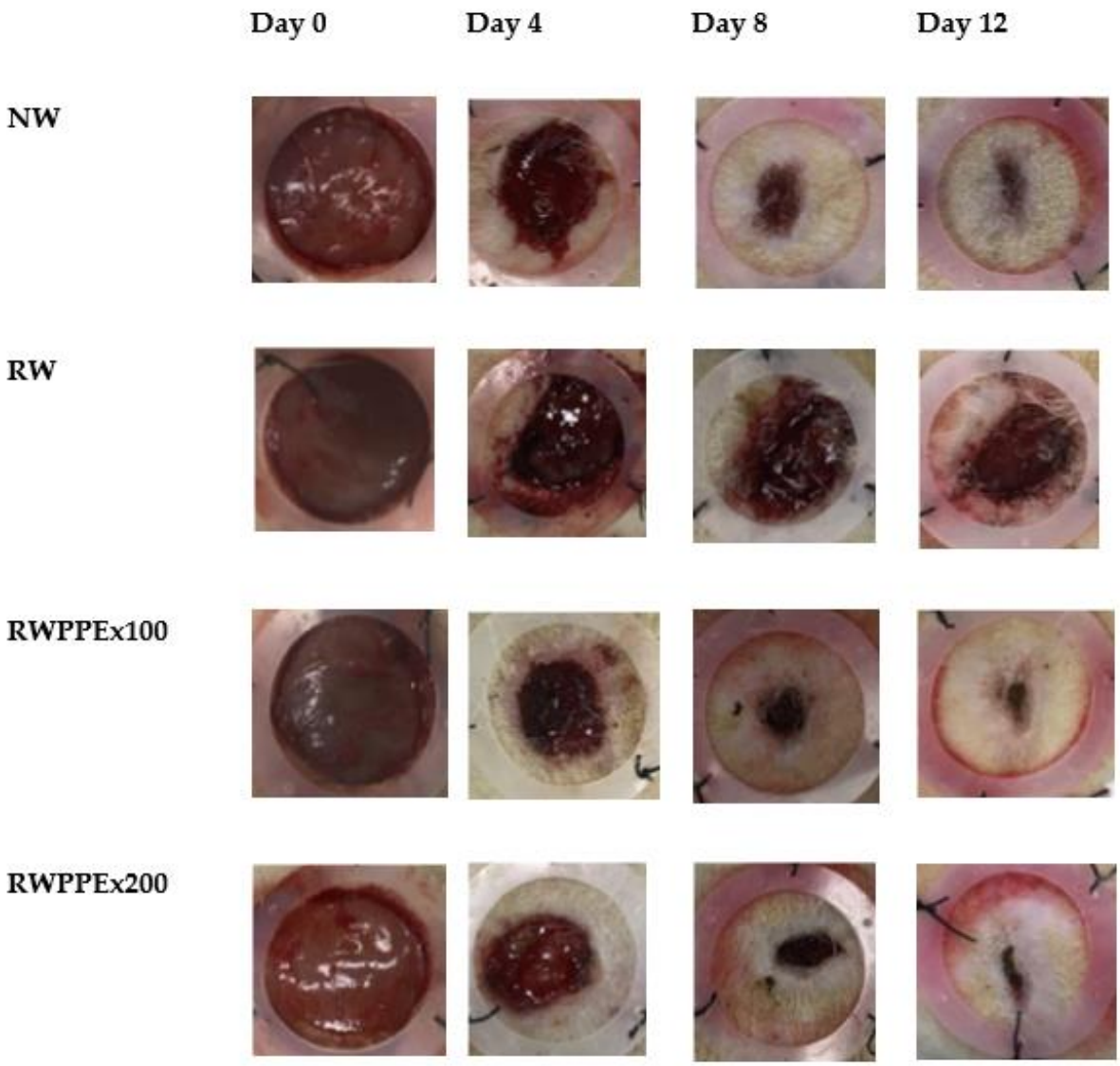

Fig. 6: Effects of PPEx on Rheumatoid arthritis wound healing in mice on day 0, day 4, day 8, and day 12 after the wound.

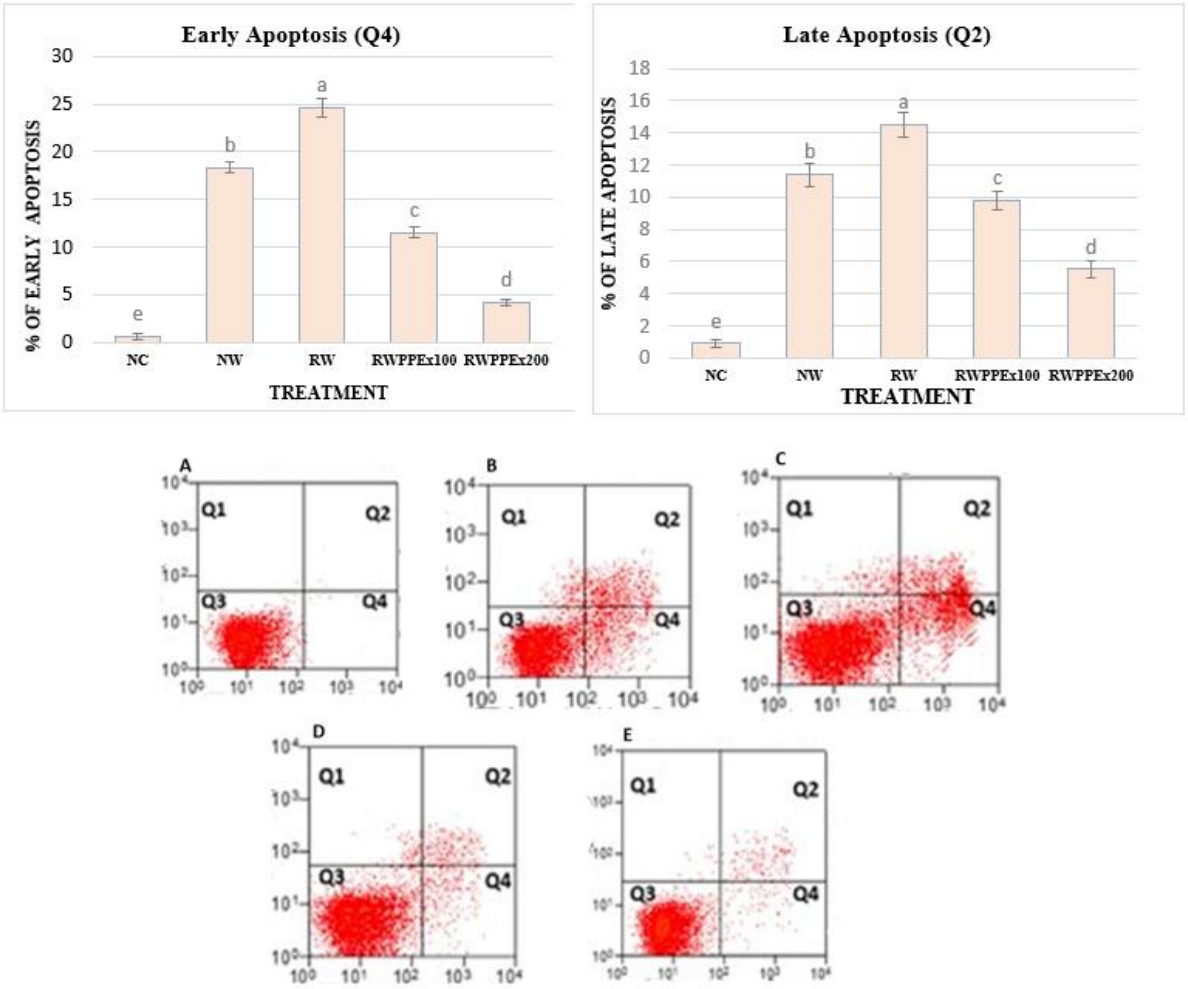

Fig. 7: Charts and histograms represent flow cytometry dot blots for untreated and PPEx-treated wounded cells. Q1: (necrotic cells), Q2: (late apoptotic cells), Q3: (viable cells) and Q4: (early apoptotic cells). Where A: normal control, B: Normal wounded mice without treatment, C: Rheumatoid wounded mice, D: rheumatoid arthritis mice with wounds treated with 100 mg/kg PPEx (RWPPEx100), and E: rheumatoid arthritis mice with wounds treated with 200 mg/kg PPEx (RWPPEx200).
Different superscript letters showed significant differences at $P< 0.05$.

Table 5: Late and early apoptosis before and after treatment with different PPEx concentrations.

Treatment	Apoptosis	
	Late apoptosis (Q2)	Early apoptosis (Q4)
N	0.9	0.6
NW	11.4	18.3
RW	14.5	24.6
RWPPEx100	9.8	11.5
RWPPEx200	5.51	4.2

Effect of the PPEx on the expression of pro-inflammatory cytokines (IL-6 and TNF-α) genes:

The investigation of cytokines gene expression levels (IL-6, TNF-α) involved in wound healing was carried out on day 14. According to Figure (8) Table (6), the qPCR findings demonstrated that the pro-inflammatory markers IL-6 (3.4, and 4.8) and TNF-α (3.5, and 4.3) were elevated in the injured NW and RW mice, respectively, in comparison to NC animals. Whereas, the PPEx treatment lowers their expression by increasing the dosage; (2.5, and 1.3) for IL-6 gene and (2.3, and 1.6) for TNF-α gene in the treatment mice RWPPEx100, and RWPPEx200, respectively.

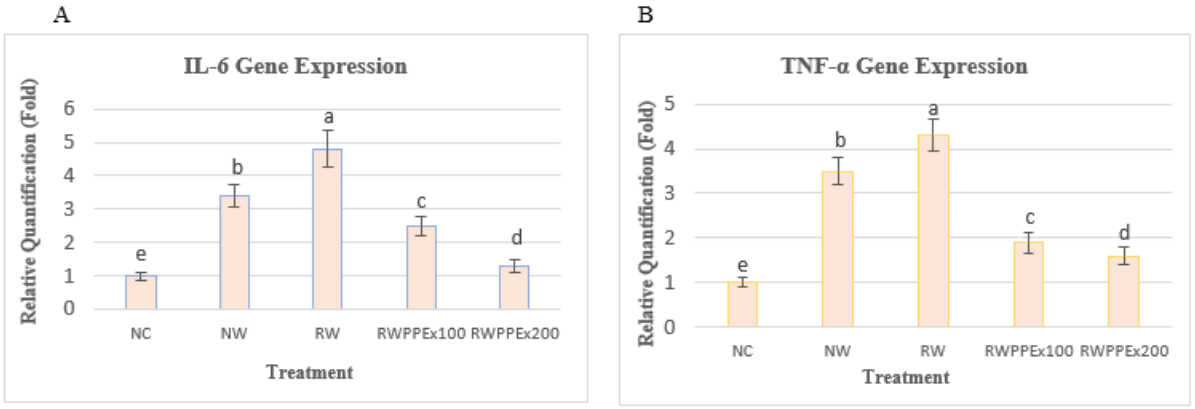

Fig. 8: Relative quantification of (A) IL-6 and (B) TNF-α gene expression
Different superscript letters showed significant differences at P< 0.05.

Table 6: Relative quantification of TNF-α, IL-6, NF-kB p65, RORϒt and T-Bet gene expression.

Gene	Treatment				
	NC	NW	RW	RWPPEx100	RWPPEx200
TNF-α	1	3.5	4.3	2.3	1.6
IL-6	1	3.4	4.8	2.5	1.3
NF-κB p65	1	3.1	4.5	2.2	1.39
RORγt	1	3.8	5.3	2.6	1.6
T-bet	1	2.4	4.3	2.1	1.3

2.5. Effect of PPEx on the expression of the transcription factors (NF-κB p65, RORγt, and T bet) genes.

On day 14, three transcription factors (NF-κB p65, RORγt, and T bet) involved in wound healing were investigated. In comparison to NC mice, injured NW (3.1, 3.8, and 2.4) or RW (4.5, 5.3, and 4.3) mice showed an increase in the expression of the transcription factors NF-κB p65, RORγt, and T-bet, respectively, according to qPCR data. Meanwhile, the PPEx therapy (RWPPEx100 (1.9, 2.1, and 1.6), and RWWPPEx200 (1.39, 1.6, and 1.3) decreases their expression by increasing the dosage (Fig. 9 and Table 6).

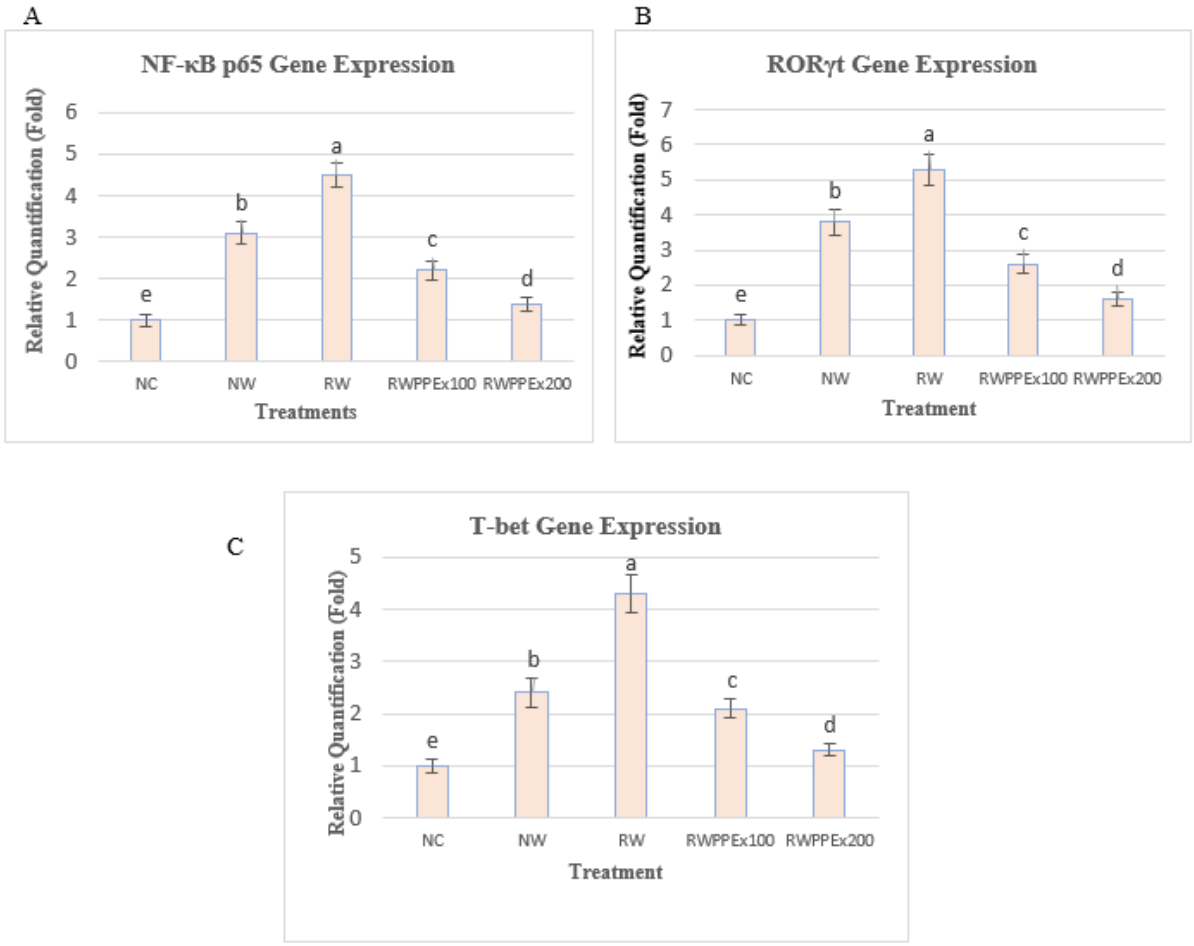

Fig. 9: Relative quantification of (A) NF-kB p65, (B) RORϒt, and (C) T-Bet gene expression Different superscript letters showed significant differences at P< 0.05.

DISCUSSION

Inflammation is a complicated defensive system of the body that may be triggered by a variety of factors, including viruses, chronic oxidative stress, trauma, exposure to toxic or irritating chemicals, wounds, and others. When exposed to a wound, immune system cells such as macrophages secrete proinflammatory cytokines, signaling proteins that cause inflammation. Chronic overproduction of these cytokines causes inflammatory disorders and should be downregulated (Sefanou *et al.*, 2020).

The present study investigated the impact of pomegranate peel extract (PPEx) on wound healing in a murine model, combining in silico and in vivo approaches. The research delved into the modulation of apoptosis levels using flow cytometry and assessed the expression of key inflammatory markers (IL-6, TNF-α, T-bet, RORγt, and NF-kB p65) via qRT-PCR to elucidate the mechanisms underlying PPEx's effects on wound healing.

Natural products are becoming increasingly important in the search for anti-inflammatory-based lead molecules for wound healing therapy (Criollo-Mendoza *et al.*, 2023 and Trinh *et al.*, 2022) Pomegranate (Punica granatum) peel is commonly used in traditional medicine. It includes several physiologically active chemicals, which account for about 30-40% of pomegranate dry weight (Poor *et al.*, 2022). Pomegranate peel is rich in polyphenols. The GC mass analysis revealed 31 active compounds in the PPEx.

Computational breakthroughs play a significant part in drug discovery, but their application to natural phytochemical substances has received less attention. Virtual screening techniques that anticipate ligand-receptor complex structures help reduce drug

development costs and time (Muthusamy *et al.*, 2011). Molecular docking is a technique for virtually identifying compounds in a library store organized by scores and structural speculative ideas, which plays an important part in structure-based drug design. The compound and the receptor play critical roles in medication formulation (Dawen *et al.*, 2024).

The Molecular docking analysis of PPEx compounds revealed promising potential for anti-inflammatory effects, particularly in the context of wound healing. The identification of seven compounds with significant binding affinities to key inflammatory mediators suggests that PPEx may offer a multi-target approach to modulating inflammation (Ismail *et al.*, 2012). Four of them (Lovastatin, Ellagitannins, punicalagin, and ellagic acid) have the potential to interact with all the 5 proteins (TNF-α, IL-6, NF-κB P65, RORϒt, and T-bet) under investigation in this research and are involved in wound healing. These results provided a theoretical foundation for the observed *in vivo* effects and highlighted potential mechanisms of action for PPEx in wound healing.

Their interaction with key inflammatory mediators can be attributed to their polyphenolic structures, which allow them to form stable interactions with protein residues, as demonstrated in docking studies (Ekowati, 2023, Hamdy, 2024).

The ADMET analysis revealed varying profiles among the compounds, which has implications for their potential therapeutic applications. Lovastatin, Pirfenidone, and Ellagic acid demonstrated favorable GI absorption and compliance with Lipinski's rule, suggesting good oral bioavailability (Lipinski *et al.*, 2001). However, the larger molecules like Ellagitannins and Punicalagin showed poor absorption properties, which may limit their systemic bioavailability. This limitation could be addressed by 1) synthesizing a series of analogs through chemical modification. 2) Prediction of biological and pharmacological properties through the structure-activity relationship. 3) Repeat the optimization cycle until the prospective candidate drug is identified (Szymański *et al.*, 2012, Leelananda and Lindert 2016).

The detailed molecular interactions observed between the PPEx compounds and target proteins provide insights into their potential mechanisms of action. The involvement of key residues in hydrogen bonding and hydrophobic interactions suggests specific and stable binding, which could lead to effective inhibition or modulation of these inflammatory mediators (Vaidyanathan *et al.*, 2023). For instance, the interactions of Ellagic acid with multiple targets through various binding modes indicate its versatility as an anti-inflammatory agent. This multi-target approach could potentially lead to more comprehensive anti-inflammatory effects compared to single-target drugs (Xie *et al.*, 2012).

The strong binding affinities of PPEx compounds to pro-inflammatory cytokines (TNF-α and IL-6) and transcription factors (NF-κB P65, RORγt, and T-bet) suggest a potential for modulating the inflammatory phase of wound healing. By potentially reducing excessive inflammation, these compounds could promote a more balanced healing process, which is crucial for optimal wound repair (Zhao *et al.*, 2016). The ability of some compounds to interact with multiple targets simultaneously could provide a synergistic effect in managing inflammation. This multi-faceted approach might be particularly beneficial in complex wound environments, such as those associated with rheumatoid conditions (Guo and Dipietro, 2010).

While this in silico study provides valuable insights, it's important to acknowledge its limitations. The predicted interactions and ADMET properties must be validated through in vitro and in vivo studies. Therefore, this research studied the expression of these inflammatory genes in RA-wounded mice.

The *in vivo* experiments demonstrated the efficacy of PPEx in promoting wound healing in the murine

model. Treatment with 200 mg/kg PPEx resulted in accelerated wound closure and improved healing outcomes, suggesting the therapeutic potential of pomegranate peel extract in tissue regeneration. This observation aligns with previous studies that have reported the wound-healing properties of pomegranate extracts (Poor *et al.*, 2022). The enhanced healing could be attributed to the high content of polyphenols, particularly ellagic acid and punicalagin, which have been shown to possess strong antioxidant and anti-inflammatory properties (Illescas-Montes *et al.*, 2024).

The flow cytometry (Annexin V) test results revealed a modulation in apoptotic activity in the PPEx-treated wounds. In our study, the early and late apoptosis were increased in the NW (18.3%, and 11.4% respectively), and RW (24.6%, and 14.5% respectively) in comparison to NC. Whereas they decrease in the RWPPEx100 (11.5% and 9.8% respectively) and the RWPPEx200 (4.2%, and 5.51% respectively) This finding suggests that PPEx may regulate the balance between cell proliferation and apoptosis, which is crucial for proper wound healing (Li *et al.,* 2020). The observed effects on apoptosis may be linked to the regulation of inflammatory cytokines and transcription factors, as evidenced by our qRT-PCR results. Apoptosis, a key mechanism in tissue healing, is crucial for removing damaged cells and promoting regeneration. In the absence of treatment, RA-wounded cells are more susceptible to imbalanced apoptosis than normal cells due to the chronic inflammatory and autoimmune processes associated with rheumatoid arthritis. Therefore, the late and early apoptosis significantly increased in the RW more than in the NW. Effective RA and wounded therapy techniques seek to address this dysregulation and promote normal apoptosis in order to enhance tissue regeneration and prevent joint degeneration (Vermes *et al.,* 1995).

Inflammation is a complicated defensive system of the body that may be triggered by a variety of factors, including viruses, chronic oxidative stress, trauma, exposure to toxic or irritating chemicals, wounds, and others. When exposed to a wound, immune system cells such as macrophages secrete proinflammatory cytokines, signaling proteins that cause inflammation. Chronic overproduction of these cytokines causes inflammatory disorders and should be downregulated (Sefanou *et al.,* 2020).

qRT-PCR analysis of key inflammatory markers and transcription factors provided insights into the molecular mechanisms underlying PPEx effects on wound healing. Our *in vivo* findings revealed that PPEx treatment affected the expression of the pro-inflammatory cytokines (IL-6 and TNF-α) by significantly decreasing their expressions than the NW and RW. This anti-inflammatory effect is consistent with previous studies on pomegranate extracts (Sefanou *et al.,* 2020) and may contribute to the accelerated healing observed in our study. Our results revealed a significant reduction in the expression of T-bet and RORγt transcription factor genes. The modulation of these T cell-specific transcription factors suggested that PPEx may influence the differentiation and activity of T helper cells during the wound healing process. This finding opens new avenues for investigating the immunomodulatory effects of PPE in wound healing (Nosbaum *et al.,* 2016).

The observed changes in NF-κB p65 expression indicate that PPEx may exert its effects through the regulation of this key transcription factor, which is known to play a crucial role in inflammation and wound healing (Ambrozova *et al*., 2017).

Finally, our study provides compelling evidence for the wound healing properties of pomegranate peel extract in mice model. The combination of in silico predictions and in vivo observations, along with the analysis of apoptosis and gene expression, offers a comprehensive understanding of PPE's effects on wound healing. These findings suggest that PPE could be a promising natural agent for promoting wound healing and managing inflammatory responses during the healing

process.

While our findings in the murine model are promising, several challenges must be addressed before translating these results to human applications. These include potential differences in pomegranate peel composition due to varietal and environmental factors, scaling of effective doses for human use, and the complex nature of human wound healing in the context of rheumatoid arthritis. Future studies should focus on standardizing PPEx preparation, conducting human cell culture experiments, and eventually progressing to clinical trials to assess the efficacy and safety of PPEx in human subjects with RA-related wound healing complications.

Conclusion:

Rheumatoid arthritis is a severe immune-mediated disorder in which the immune system of the host destroys the healthy tissues in the body, —moreover, patients may encounter complications like vasculitis, ulceration, and delayed wound healing. The findings of our study suggest that PPEx could be developed as potential inhibitors that can target TNF-α, IL-6, NF-κB p65, RORγt, and T bet proteins. Through the *in-silico* studies, significant insights were gained that could prove useful for the further development of novel inhibitors for wound healing in patients with rheumatoid arthritis.

The current investigation discovered that administering PPEx at a dosage of 200 mg/kg accelerated cell growth and dramatically improved wound healing. This work sheds light on how PPEx reduces the expression of genes such as IL-6, TNF-α, NF-κB p65, RORγt, and T bet. Finally, these findings established a foundation for future study and the use of PPEx to accelerate wound healing.

Our *in silico* analysis identified several active compounds in PPEx with potential anti-inflammatory and wound healing properties. Future research should investigate the possible synergistic effects of these compounds, particularly Lovastatin, Ellagitannins, Punicalagin, and Ellagic acid, which showed interactions with all five proteins of interest. Combination studies using purified compounds in various ratios could reveal optimal formulations for enhancing wound healing efficacy. Additionally, exploring the potential synergy between PPEx and conventional RA treatments could lead to novel combination therapies for managing both RA symptoms and associated wound healing complications.

Declarations:

Ethical Approval: Ain Shams University Committee on Experimental Animal Care and Studies Ethics, Agriculture Sector Committee, authorized all studies involving the use of animals (permission No. 15-2024-03).All procedures involving animals were performed following the guidelines established by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The welfare of the animals was prioritized throughout the study, ensuring minimal distress and pain.We affirm that all personnel involved in the study were trained in the proper handling and care of animals and that all efforts were made to reduce the number of animals used in accordance with the principles of the 3Rs (Replacement, Reduction, and Refinement).By adhering to these ethical standards, we aim to ensure the integrity of our research and the humane treatment of all animals involved.

Conflict of interests: The authors declare no conflicts of interest.

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Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

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