

ASSESSMENT OF THE ANTI-ARTHRITIC AND IMMUNOSUPPRESSIVE POTENTIAL OF ENALAPRIL BY USING NETWORK PHARMACOLOGY, MOLECULAR DOCKING AND EXPERIMENTAL PHARMACOLOGY APPROACHES

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Abstract

The present study validates the anti-arthritis and immunosuppressive attributes of enalapril, an angiotensin-converting enzyme inhibitor (ACEI) with a potential role in major inflammatory pathways. A network pharmacology-based prediction approach was utilised to reveal the possible target genes that can be targeted by enalapril for managing arthritis. *In vitro* anti-arthritis efficacy of enalapril was assessed using an assay that measures the denaturation of proteins in bovine serum albumin (BSA), egg albumin and the stability of the human red blood cell (HRBC) membrane at a dosage ranging from 100 to 6400 µg/mL. *In vivo* anti-arthritis efficacy of enalapril at doses of 2, 4 and 8 mg/kg bw was assessed in formaldehyde-induced arthritis model. Dinitrochlorobenzene (DNCB)-provoked delayed type hypersensitivity (DTH) and cyclophosphamide-provoked myelosuppression were employed to assess the immunosuppressant capacity of enalapril. Network pharmacology outcomes revealed that the anti-arthritis effects of enalapril targets include tumour necrosis factor (TNF), matrix metalloproteinase 9 (MMP-9) and caspase 3 (CASP3). Molecular docking of enalapril with these three targets also validates the strong interaction between them. Enalapril markedly inhibited protein denaturation in egg albumin and bovine serum albumin (BSA) assays and stabilised RBC haemolysis exposed to hypotonic media. Likewise, enalapril demonstrated a dose-dependent inhibition of paw oedema provoked by formaldehyde. In the DTH assay, enalapril significantly reduced skin thickness compared to the negative control group and exhibited potent immunosuppressant potential in cyclophosphamide-induced myelosuppression. Based on the outcomes of the current study, it can be predicted that enalapril has anti-arthritis attributes that might be due to its immunosuppressive potential.

Rezumat

Studiul validează efectele antiartrite și imunosupresoare ale enalaprilului, un inhibitor al enzimei de conversie a angiotensinei (IECA) cu potențială acțiune în patologiile inflamatorii. Prin intermediul unei abordări farmacologice predictive s-au identificat potențialele gene țintă ale enalaprilului în terapia artritei. Efectul *in vitro* al enalaprilului a fost evaluat prin testul de denaturare a albuminei serice bovine (BSA), al albuminei din ou și de stabilizare a membranei globulelor roșii umane (HRBC) la o doză cuprinsă între 100 și 6400 µg/mL. Acțiunea *in vivo* a enalaprilului în doze de 2, 4 și 8 mg/kgc a fost evaluată în artrita indusă experimental cu formaldehidă. Pentru a evalua acțiunea imunosupresoare a enalaprilului a fost utilizat testul hipersensibilității întârziate (DTH), folosind dinitroclorobenzen (DNCB) și mielosupresia provocată de ciclofosamidă. Rezultatele abordărilor farmacologice au arătat că efectul antiartritic al enalaprilului este atribuit interacțiunii cu factorul de necroză tumorală (TNF), metaloproteinaza 9 a matricei (MMP-9) și caspaza 3 (CASP3).

Keywords: arthritis, network pharmacology, molecular docking, cyclophosphamide egg albumin, enalapril

Introduction

Rheumatoid arthritis (RA) is a complex condition characterised by joint discomfort, inflammation and synovial tissue proliferation. These factors contribute to joint degeneration and functional impairment [1, 2]. The joint deterioration in RA is attributed to the overexpression of cytokines and transcription factors.

The interleukins, notably IL-6, IL-1, IL-17 and tumour necrosis factor (TNF), are cytokines having a clear role in RA [1]. IL-6 promotes blood vessel development, which in turn causes inflammation [3]. TNF- α increases inflammation by stimulating synovial fibroblasts to produce cellular adhesion molecules and promoting leukocyte movement towards joints [4]. Prostaglandin

(PGE2) and nitric oxide (NO) synthesis are both modified by IL-1, which also aids in the degradation of cartilage and the resorption of bones [5]. The imbalance between proinflammatory and anti-inflammatory mechanisms causes damage to the joint's synovial membrane, resulting in joint inflammation and damage. The five different types of medications that can be used to treat RA include steroid hormones, immunosuppressant biological agents, anti-rheumatic drugs and anti-inflammatory medications [6]. Despite the fact that anti-arthritis drugs have a number of benefits, their clinical use is constrained by their high cost, unfavourable side effects and restricted efficacy for a specific target location [7].

ACEIs are commonly employed for the management of congestive heart failure, ischemic heart disease and renal disease [8]. The ACE enzyme catalyses the breakdown of substance P and bradykinin and converts angiotensin I to angiotensin II. Angiotensin II, a potent vasoconstrictor, is widely used to regulate blood pressure, vascular tone and extracellular volume. Apart from the mentioned attributes, angiotensin II offers a range of other effects, most notably autocrine and paracrine proinflammatory characteristics [9]. Dendritic cells and monocytes/macrophages in the synovial fluid produce angiotensin II *via* ACE [10]. Angiotensin II signalling *via* the AT1 receptor activates the transcription factor nuclear factor- κ B (NF- κ B) [11] that produces reactive oxygen species, pro-inflammatory cytokines and adhesion molecules. Thus, inhibiting ACE and NF- κ B activation will prevent the proinflammatory cytokines (IL-6, TNF- α and IL-1) produced by monocytes and dendritic cells [12].

Additionally, RA disturbs the renin-angiotensin system. Patients with RA have been shown to have elevated ACE activity in their synovial tissue, blood monocytes, synovial fluid and nodules [13]. Previous studies also demonstrated the anti-arthritis potential of other ACEIs. One of the studies conducted on captopril to predict its anti-arthritis potential revealed promising effects of captopril against complete Freund's adjuvant-induced arthritis and hypersensitivity assay in rats [14]. Quinapril was also tested for its ability to prevent arthritis in a collagen-induced arthritis model. It exhibited significant anti-inflammatory efficacy in alleviating collagen-induced arthritis symptoms [15]. Moreover, ramipril also demonstrated significant anti-arthritis potential in CFA-induced arthritis by normalising the levels of inflammatory mediators involved in RA pathophysiology [16]. Thereby, in the present study, the anti-arthritis and immunosuppressant potential of enalapril was evaluated to predict its possible role in RA, as enalapril has documented anti-inflammatory potential [17].

In the time of targeted drug discovery, network pharmacology aims to comprehend pharmacological effects as well as interactions with various targets. The advancement of network pharmacology provides

hope for drastically changing the method of developing drugs in the future. Building a "compound-protein/gene-disease" network using network pharmacology is considerably more feasible and allows for high-throughput discovery of the unlimited potential of biologically active compounds. This is the first study to use network pharmacology to look at the anti-arthritis potential of enalapril. This study clarifies the molecular mechanism underlying the anti-RA effects of enalapril and may expedite the drug discovery process. Additionally, laboratory research was conducted to examine the fundamental principles underlying the anti-arthritis effect of enalapril.

Materials and Methods

Network Pharmacology

Target genes screening of RA and enalapril

The target genes for RA were retrieved from DisGeNET [18], while the target genes for enalapril were accessed through binding DB [19] and Swiss target prediction [20].

Protein interaction (PPI) network construction

STRING 11.0 is a web-based database that can gather, evaluate and combine data on protein-protein interactions from all publicly accessible sources. *Homo sapiens* was chosen as the species, and a minimum interaction score of 0.7 was used to construct a protein interaction network. The results were imported into Cytoscape 3.7.2 for visual examination.

KEGG pathway analysis

To clarify the function of target proteins that interact with enalapril, the KEGG pathway enrichment of proteins included in the PPI network was examined using the Database for Annotation, Visualisation and Integrated Discovery [21] version 6.8.

Molecular Docking

MOE 2015 was employed to conduct docking studies. A 3D model of enalapril was created in MOE. Energy minimisation was done, and formal charges on atoms were verified by the 2D representation. The partial charges were determined automatically. The 3D crystal structure of TNF, MMP-9 and CASP3 was retrieved from the protein data bank with PDB IDs 2AZ5, 1GKC and INME. The crystal structures were initially protonated, hydrogen atoms were added, and automatic correction was used to check for any errors. The MOE tool's energy minimisation algorithm was used to reduce the energy of the protein molecule. The following variables were used to minimise energy: 0.05 Gradient, MMFF94X, Solvation Force Field, and Current Geometry Chiral Constraint. When the root mean square gradient dropped below 0.05, energy minimisation was stopped. Using all of the default parameters of Site Finder, the co-crystallised inhibitor's active site was chosen and dummy atoms for the pocket were made. Enalapril was docked into the binding pocket of selected targets and ten different poses of

enalapril with each target were obtained. The poses obtained after completing the docking procedure were evaluated to identify the ones with optimal ligand-target interactions and acceptable RMSD values. These selected poses were then saved for subsequent energy calculations [22].

Animals

Sprague Dawley rats (200 - 300 g, any sex) were utilised in this study. They were housed in stainless steel cages and provided with a free diet and unlimited access to water. The rats were kept in a 12-hour light/dark cycle in accordance with National Institutes of Health (NIH) recommendations for the care and management of laboratory animals. The Institutional Animal Ethics Committee, Faculty of Pharmacy, University of Sargodha, Pakistan, accepted the study protocol. The guidelines of the National Research Council were followed for all the experiments [23].

HRBC membrane stabilisation assay

Following the explanation of the study's objectives and procedures, informed consent was obtained from

$$\% \text{age inhibition} = 100 - \left[\left(\frac{\text{absorbance sample}}{\text{absorbance control}} \right) \times 100 \right].$$

Egg albumin induced protein denaturation assay

The reaction mixture (5 mL) contained 2 mL of piroxicam and enalapril at various concentrations (100, 200, 400, 800, 1600, 3200 and 6400 g/mL, respectively), as well as egg albumin (0.2 mL) and phosphate-buffered saline (2.8 mL). Double-distilled water serves as the

$$\% \text{age inhibition} = \left[\frac{\text{Absorbance control} - \text{Absorbance test sample}}{\text{Absorbance test control}} \right] \times 100.$$

Bovine serum albumin (BSA) induced protein denaturation assay

The reaction mixture (0.5 mL) contains 0.05 mL of varying concentrations of piroxicam (the reference drug) and enalapril (100, 200, 400, 800, 1600, 3200 and 6400 g/mL, respectively). The samples were heated for 30 minutes at 57°C after 20 minutes of

$$\% \text{age inhibition} = 100 - \left[\frac{\text{absorbance test solution} - \text{absorbance product control}}{\text{absorbance test control}} \right] \times 100.$$

Formaldehyde induced arthritis

A sub-plantar injection of 2% formaldehyde solution (0.1 mL), given 30 minutes after the administration of the drug, was used to induce arthritis on day 1 and a second induction on day 3 of the experiment. For

$$\% \text{age inhibition} = \left[\frac{\text{Paw volume of control group} - \text{Paw volume of test group}}{\text{Paw volume of control group}} \right] \times 100.$$

The rats (n = 6) were divided into five groups. The rats with arthritis were subsequently categorised into different groups according to the medications administered. Group I was administered a dose of 3 mL/kg bw of distilled water; Group II received a dose of 10 mg/kg bw of piroxicam; and Groups III, IV and V were given doses of 2, 4 and 8 mg/kg bw of enalapril orally.

Dinitrochlorobenzene (DNCB) induced delayed type hypersensitivity (DTH) assay

Six groups, each consisting of six mice, were established. Groups I and II were administered with vehicle only, specifically 3 mL/kg of distilled water, and served as control groups, with Group I being the negative control

the participants. Subsequently, a 10 mL blood sample was collected from the healthy participant. The Institutional Human Ethics Committee of the Faculty of Pharmacy, University of Sargodha, Pakistan, approved the study. After that, blood was spun at 3000 rpm in a centrifuge with an equal volume of sterilised Alsevers solution. Following isosaline-washing of packed cells, a 10% v/v solution of red blood cells was prepared. The test solution contained 0.5 mL of enalapril at different concentrations (100, 200, 400, 800, 1600, 3200 and 6400 g/mL), 0.5 mL of 10% HRBC and 1 mL of phosphate buffer (pH 7.4, 0.15 M). One mL of 10% HRBC in isotonic saline, 2 mL of distilled water and 1 mL of phosphate buffer made up the test control solution. The haemoglobin concentration was determined spectrophotometrically at 560 nm after assay mixtures were centrifuged at 3000 rpm for 30 minutes while being incubated at 37°C [24]. The formula shown below was used to estimate the percentage of membrane stability:

control. The mixtures were heated at 70°C for 5 minutes after incubating at 37 ± 2°C for 15 minutes. At 660 nm, the absorbance was measured [25]. Using the following formula, the percentage of protein denaturation inhibition was calculated:

incubation at 37°C. After that, 2.5 mL of phosphate buffer was added, and a spectrophotometer was used to detect the absorbance at 660 nm. While the product control lacked BSA, the test control used 0.05 mL of distilled water instead of the drug [26]. By using the formula below, the inhibition of protein denaturation was calculated:

ten days, there was continuous drug treatment. Using a digital plethysmometer, the average rise in paw volume over ten days was checked to assess arthritis [27]. The following formula was employed for the calculation of the percentage inhibition of paw oedema:

and Group II being the positive control. Groups III, IV and V were orally administered enalapril at dosages of 2, 4 and 8 mg/kg bw, respectively, dissolved in the vehicle. As a reference medication, an immunomodulator was administered to Group VI at a dose of 0.1 mL/kg. The ingredients for the immunomodulator were 1 g sodium selenite, 15 g vitamin E, 9 g sodium chloride and distilled water *q.s.* to 1000 mL.

An eight-day treatment period included the administration of enalapril and an immunomodulator. Groups of mice were neatly shaved for the DTH testing. On day 2, all mice were sensitised with 0.1 mL of 2% DNCB, except for the negative control group, which received

a sham procedure using 0.1 mL of acetone alone. Before the DNCB application, the mice's skin thickness was measured using a vernier caliper. Except for the negative control group, which was falsely challenged with 0.2 mL of acetone on day 8, sensitised mice were retested with 0.2 mL of 2% DNCB on that day. At 24, 48 and 72 hours following the DNCB test, the skin's thickness was measured [28].

Cyclophosphamide induced myelosuppression

Six groups of six mice each were created. Groups I and II were treated with only vehicle (3 mL/kg bw distilled water) and acted as control groups (negative and positive). Low, medium and high (2, 4 and 8 mg/kg bw) dosages of enalapril dissolved in the vehicle were administered orally to Groups III, IV and V. As a reference medication, an immunomodulator was administered to Group VI at a dose of 0.1 mL/kg bw. The ingredients for the immunomodulator were 1 g sodium selenite, 15 g vitamin E, 9 g sodium chloride and distilled water *q.s.* to 1000 mL.

Enalapril and an immunomodulator were administered to mice in the experimental groups over ten consecutive days. On the tenth day, cyclophosphamide was subcutaneously injected at a 200 mg/kg bw dose. Before and 72 hours after the cyclophosphamide injection,

blood samples from rat tails were taken. Using an automated hemacytometer, the total number of white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), lymphocyte and neutrophil levels were all determined [29].

Statistical Analysis

The data was presented as mean \pm SEM, and statistical analysis was conducted using one-way ANOVA followed by Dunnett's test and two-way ANOVA followed by the Bonferroni post-test in GraphPad Prism 8.0.2. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Network Pharmacology

Identification of disease related and drug related targets

A Swiss target prediction database was employed for predicting enalapril's pharmacological targets. Among all targets, we selected those targets that had at least 50% similarity with enalapril. The names of 49 targets for enalapril are mentioned in Table I. Likewise, 2722 Ra-related genes were retrieved from DisGeNET. The overlapping genes between enalapril and RA were found by using a Venn diagram, as shown in Figure 1.

Table I

Binding mode analysis of enalapril with selected targets

Targets	Docking Score (kcal/mol)	RMSD-Refine	Amino acid Residue/Bond	Distance
TNF	-5.749	1.5122	Lys89	2.93
			Lys89	3.29
MMP-9	-8.1798	1.8305	His401	--
			Tyr423	
CASP3	-5.8508	1.1066	Arg207	3.09

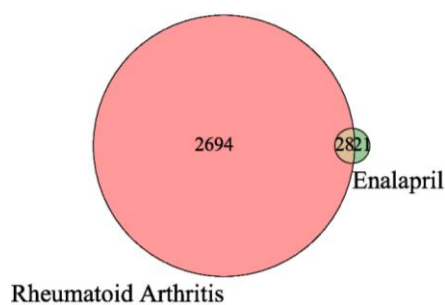


Figure 1.

A Venn diagram demonstrating the number of overlapping genes between enalapril and RA. The red colour circle represents RA genes, the green colour represents enalapril targets and the yellow colour represents intersecting genes between RA and enalapril

Construction of PPI

28 overlapping genes were screened further for protein-protein interactions by submitting them to the String database. In the PPI network, nodes and their associated interactions indicate the interrelationship among multiple targets during disease development (Figure 2A). The CytoHubba plugin tool from Cytoscape was then used

to analyse the PPI network. There are 12 topological parameters in CytoHubba, and among them, we used the degree method for calculating the interactions of the nodes. The highest degree indicates a strong correlation of target genes with one another, making all of these genes potentially important targets. According to the results of the degree-based analysis, TNF (22), MMP9 (18), CASP3 (16), SIRT1 (15), PTGS2 (14), CTSB (12), MMP3 (11), MMP2 (11), REN (10) and CASP8 (9) were deemed to be the top ten hub genes (Figure 2B). After comparing the findings of PPI analysis with enrichment analysis (Table II), three genes, particularly TNF, CASP3 and MMP9, appeared to be the key anti-arthritic targets of enalapril.

KEGG Analysis

To identify the relevant signalling pathways associated with the anti-arthritic impact of enalapril, a KEGG pathway investigation was performed. The pathways with the highest number of genes are the apoptosis pathway, the IL-17 signalling pathway and the TNF signalling pathway. Also, the results of the KEGG pathway analysis showed that the genes CASP3, PPARA, MMP9 and MMP3 were considerably enriched in the top ten pathways (Figure 3).

Table II

Inhibitory impact of enalapril on formaldehyde induced paw oedema

Experimental groups	Paw volume (mL)				
	Day 2	Day 4	Day 6	Day 8	Day 10
Disease control (3 mL/kg)	1.556 ± 0.047	1.784 ± 0.046	1.731 ± 0.024	1.663 ± 0.058	1.683 ± 0.054
Piroxicam (10 mg/kg)	1.168 ± 0.035*** (24.93%)	1.013 ± 0.078*** (43.21%)	0.653 ± 0.017*** (62.27%)	0.565 ± 0.033*** (66.02%)	0.472 ± 0.045*** (71.95%)
Enalapril (8 mg/kg)	0.848 ± 0.021*** (45.50)	1.020 ± 0.028*** (42.82%)	0.660 ± 0.027*** (61.87%)	0.548 ± 0.059*** (67.04%)	0.345 ± 0.084*** (79.50%)
Enalapril (4 mg/kg)	1.052 ± 0.056*** (32.39)	1.110 ± 0.061*** (37.78)	0.842 ± 0.040*** (51.35%)	0.633 ± 0.053*** (61.93%)	0.500 ± 0.035*** (70.29%)
Enalapril (2 mg/kg)	0.972 ± 0.057*** (37.53)	1.117 ± 0.064*** (37.38%)	0.875 ± 0.035*** (49.45%)	0.677 ± 0.051*** (59.89%)	0.547 ± 0.061*** (67.49%)

Data is represented as mean ± SEM (n = 6). When comparing the results to the arthritic control group, p < 0.005 was deemed statistically significant, while ***p < 0.001, **p < 0.01, *p < 0.05

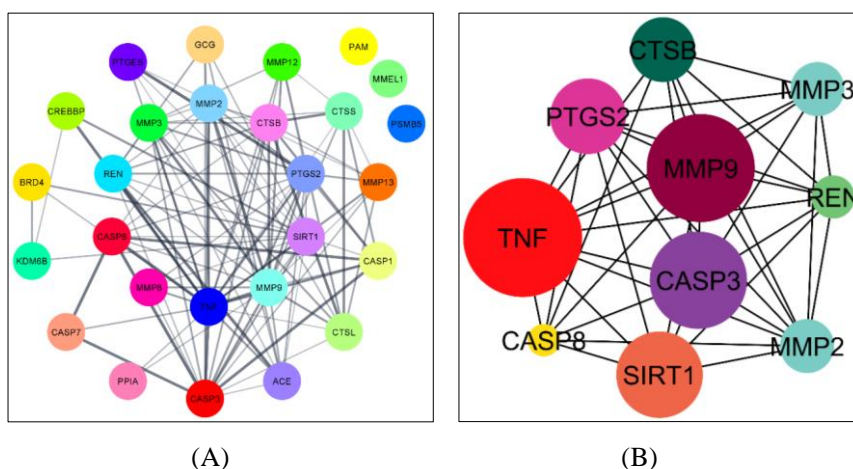


Figure 2.

(A) PPI network of 28 overlapped genes; (B) Top ten genes in the PPI network according to the degree method. Size and colour of circle represents their degree value in the network

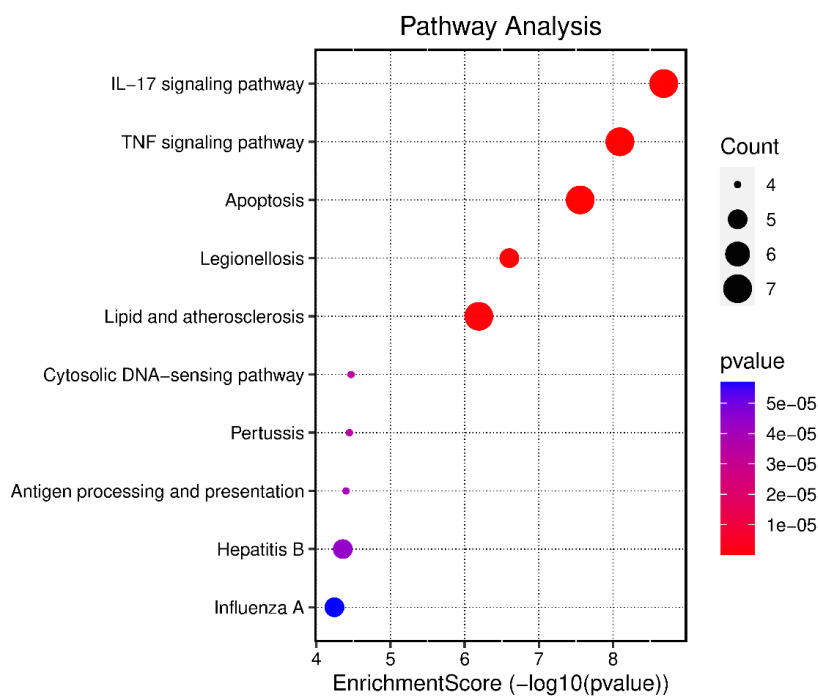


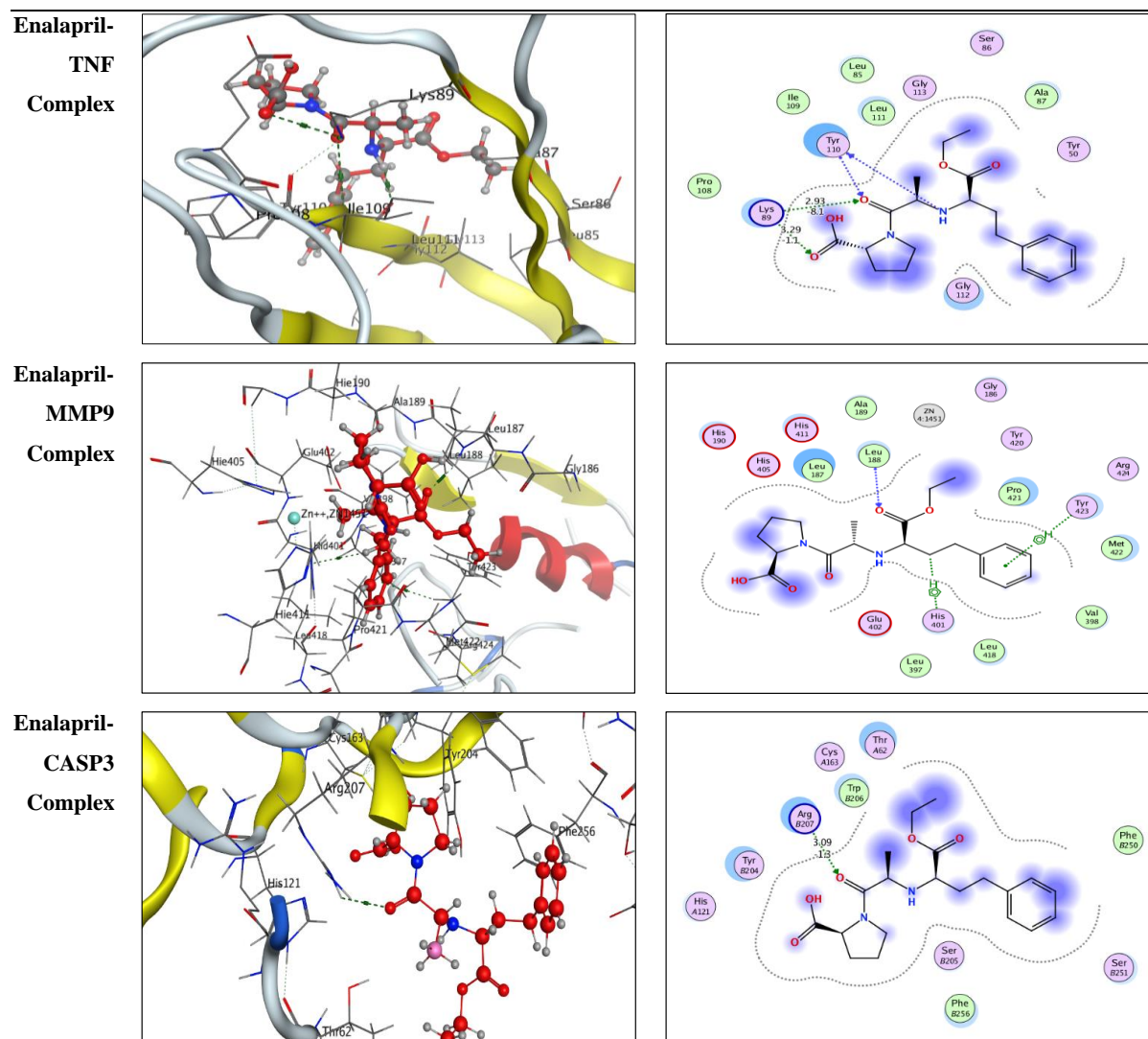
Figure 3.

KEGG pathway analysis of overlapping genes

Molecular docking

Molecular docking of enalapril was performed with selected targets, including TNF, CASP3 and MMP9. Docking analysis of the compound revealed that enalapril occupies the binding pocket of selected targets. With TNF, enalapril demonstrated two H-bond interactions with Lys89. Binding mode analysis of enalapril with TNF displayed a score of -5.749 kcal/mol and an

RMSD of 1.5122. With MMP-9, enalapril demonstrated a score of -8.1798 and 1.8305 RMSD, while the binding mode pattern revealed two H-pi with His401 and Tyr423. Also, enalapril with CASP3 shows H-bond interaction with Arg207 with a score of -5.8508 and an RMSD of 1.1066, as shown in Table I and Figure 4.

**Figure 4.**

3D and 2D representations of the binding mode of enalapril with selected targets

Anti-arthritic evaluation of enalapril via HRBC membrane stabilization

The outcomes of the HRBC membrane stabilisation assay revealed that enalapril has a positive impact on stabilising the RBC membrane. This response was concentration-dependent. A maximum response of $73.39 \pm 1.051\%$ was observed at a concentration of $6400 \mu\text{g/mL}$. These outcomes were comparable to those of the piroxicam, which also showed a concentration-dependent stabilisation of HRBC, with the highest effect at $6400 \mu\text{g/mL}$, as shown in Figure 5.

Anti-arthritic evaluation of enalapril via protein denaturation inhibition

Enalapril's ability to prevent protein denaturation was assessed using the egg albumin and BSA denaturation assays. Enalapril significantly inhibited protein denaturation in both egg albumin and the BSA assay in a concentration-reliant manner, with the maximum response at $6400 \mu\text{g/mL}$ and the minimum at $100 \mu\text{g/mL}$ (Figure 6).

HRBCs membrane stabilization

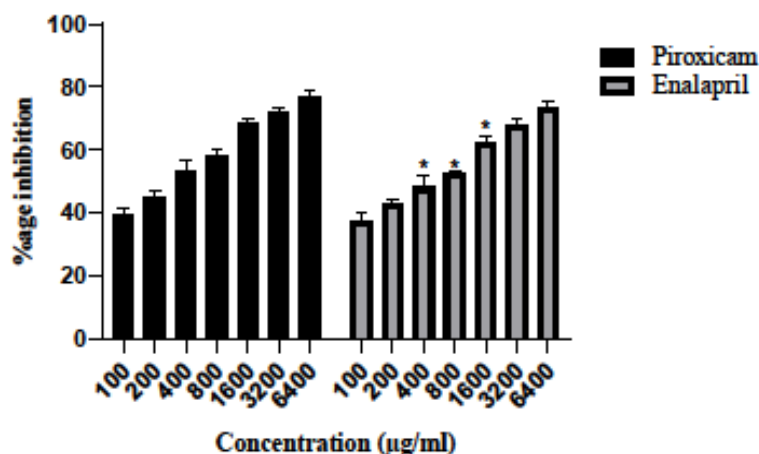


Figure 5.

The impact of enalapril on preventing RBC haemolysis brought on by exposure to hypotonic saline medium Data is evaluated using a two-way ANOVA and then the Bonferroni post-test. Values are given as mean ± SEM (n = 3)

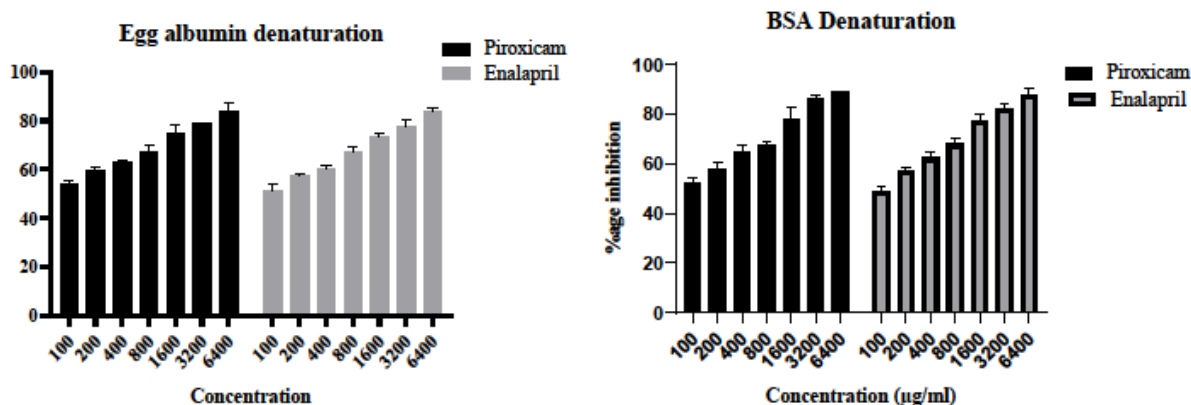


Figure 6.

Effect of Enalapril on inhibition of RBC haemolysis due to exposure to hypotonic saline media Data are evaluated using a two-way ANOVA and then the Bonferroni post-test. Values are given as mean ± SEM (n = 3)

Anti-arthritis evaluation of enalapril via formaldehyde-induced arthritis assay

The anti-arthritis efficacy of enalapril was estimated using a formaldehyde-prompted arthritic model. Three different doses of enalapril (2, 4 and 8 mg/kg) were administered to experimental animals, and paw volume was assessed for ten days. Enalapril displayed dose-dependent inhibition of paw volume augmentation, the most significant effect being observed at the dose of 8 mg/kg. Compared to the arthritic control group, a 79.50% inhibition of paw volume increase was demonstrated by enalapril 8 mg/kg on the 10th day of the study. All doses of enalapril produced a highly significant suppression of paw volume compared to the disease control group. Piroxicam, used as a standard drug, also showed highly significant paw volume suppression with a percentage inhibition of 71.95% on the 10th day, as shown in Table II.

Enalapril induced suppression of delayed type hypersensitivity

After 24 hours of induction, all treated groups that received DNCB had thicker skin. As compared to the negative control group (0.017 ± 0.03), there was a marked increase in the skin thickness among the positive control animals (1.6 ± 0.05, p < 0.001). Enalapril administration to experimental animals at low, medium and high doses resulted in highly significant inhibition of the increase in skin thickness when compared with the positive control group after 24 hours. The same pattern of skin thickness increase was observed after 48 hours and 72 hours in positive group animals (1.217 ± 0.03 and 0.883 ± 0.05). This shows a highly significant increase in skin thickness when compared with animals included in the negative control. On the contrary, all doses of enalapril demonstrated suppression of DNCB-induced skin thickness after 48 and 72 hours in a dose-dependent manner when compared with the positive control group, as presented in Figure 7.

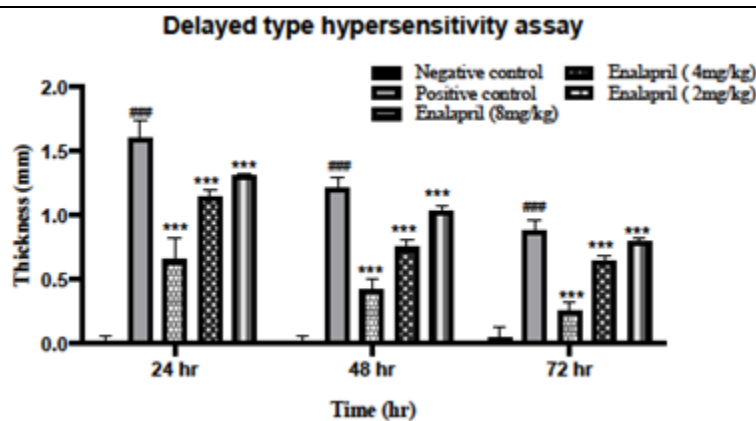


Figure 7.

Enalapril treatment resulted in significant suppression of delayed-type hypersensitivity induced by DNCB. Data is represented as mean \pm SEM (n = 6); *** p < 0.001 represents comparison between enalapril-treated and positive control groups; ### p < 0.001 represents comparison between the positive and negative control groups

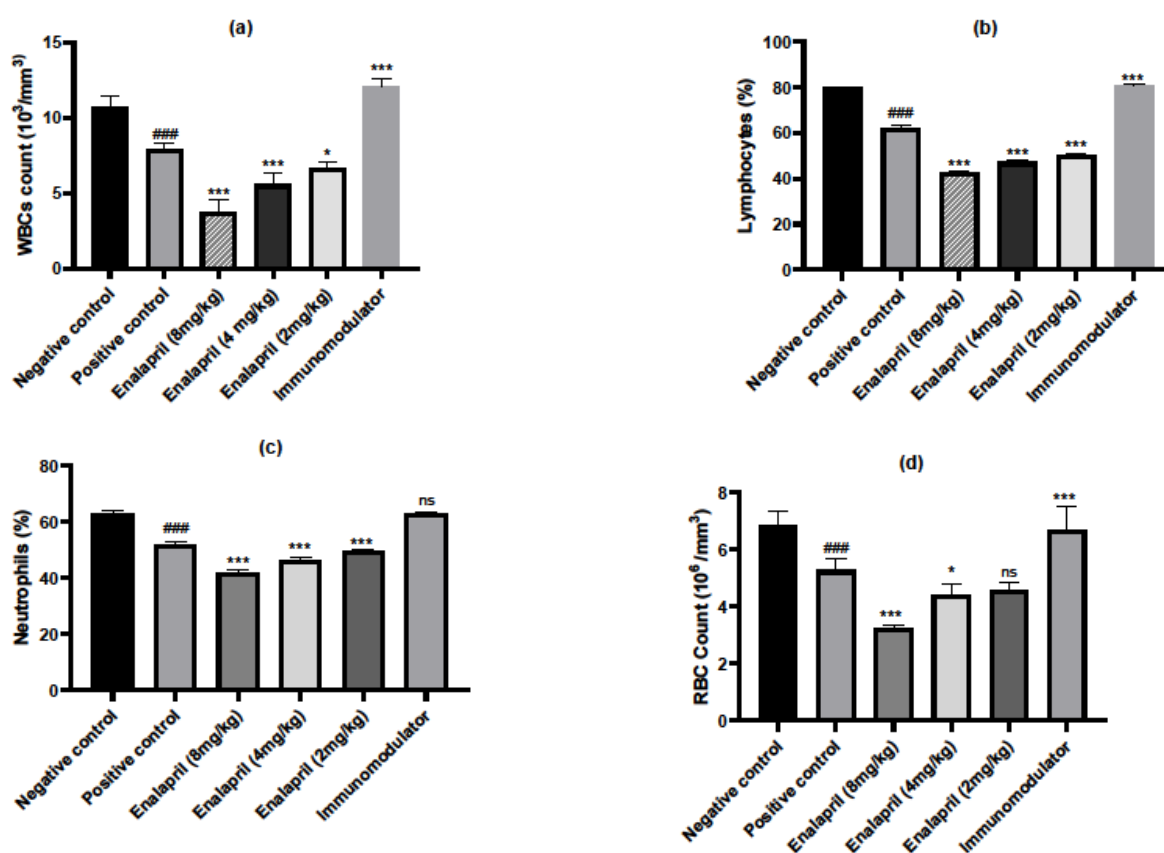


Figure 8.

Enalapril treatment resulted in significant suppression of (a) white blood cell count, (b) lymphocyte count, (c) neutrophil count, (d) red blood cell count

Data is represented as mean \pm SEM (n = 6); *** p < 0.001, ** p < 0.01, * p < 0.05 represents comparison between enalapril treated and positive control groups; ### p < 0.001 represents comparison between positive and negative control groups

Protective implication of enalapril against cyclophosphamide myelosuppression

The positive control group showed a significant decrease in leukocyte count (7.90 ± 0.157) compared to the negative control animals (10.75 ± 0.301). A significant attenuation in WBC count was obtained among experimental groups provided with low (6.643 ± 0.19), medium (5.58 ± 0.32) and high (3.76 ± 0.33) doses

of enalapril when compared to positive control animals. The immunomodulatory treatment significantly prevented cyclophosphamide-induced myelosuppression (12.05 ± 0.22), as shown in Figure 8(a).

Lymphocyte count, as compared to positive control animals (62.080 ± 0.453), was significantly reduced in the enalapril-provided group in a dose-dependent manner (42.70 ± 0.225 , 47.17 ± 0.287 , $50.230 \pm$

0.232). Likewise, compared to the positive control group, enalapril, at all three doses, significantly reduced neutrophils and RBC count in a dose-dependent manner, as shown in Figure 8(c). The neutrophil count (%) displayed by three doses (high, medium and low) of enalapril was found to be 41.92 ± 0.52 , 46.47 ± 0.33 and 49.49 ± 0.28 , respectively.

Rheumatoid arthritis (RA) is an autoimmune disorder characterised by multiple pathobiological processes, although the precise underlying mechanism remains uncertain. A lot of work has been done regarding its pathophysiological exploration and management strategies [30]. However, research is still going on in this field to explore a potential treatment option with efficacy and fewer side effects. Accumulating evidence advocates that disease characteristics and traditional risk factors equally contribute to enhanced CVD risk in RA [31]. The discovery of efficient strategies to lower this RA-associated CVD risk is now a main focus of research. Preventative interventions to suppress both inflammation and CVD risk in RA are of prime interest to combat this mortality rate. As a result, RA patients need novel therapeutic strategies and means to combat the risk of CVD, thereby targeting the angiotensin system, notably angiotensin II (Ang II) and its receptors, which might be one of those novel approaches [15]. Thereby, in the current research, we made an attempt to explore the anti-arthritis and immune suppressant potential of enalapril.

Network pharmacology-based analysis revealed 28 intersecting genes between enalapril and RA targets. These genes were analysed further in the PPI and KEGG pathways. Based on the outcomes of both analyses it was concluded that four genes, particularly CASP3, PPARA, MMP9 and MMP3, revealed significant interactions with other proteins in the PPI network, and these proteins were also enriched in KEGG pathway analysis. Among the top ten pathways being targeted by overlapping genes in the KEGG analysis two highly enriched pathways, the IL-17 signalling pathway and the TNF signalling pathway, were of particular interest as these have an evident role in arthritis. Both early and advanced RA symptoms are affected by IL-17, which promotes the activation of fibroblasts like synoviocytes (FLS), osteoclastogenesis and the recruitment and activation of neutrophils, macrophages and B cells [28]. Studies have demonstrated that the interaction of IL-17 and TNF- α increases the production of pro-inflammatory mediators such as IL-1, IL-6, IL-8, PGE2 and matrix metalloproteinase (MMPs), which accelerates the progression of early inflammation into chronic arthritis [32, 33]. Molecular docking studies further validate that enalapril can target TNF, MMP9 and CASP3 and have considerable binding interactions with selected targets.

According to these findings, the anti-arthritis immunosuppressive effects of enalapril were found to be dose-dependent. The present study revealed concentration-

reliant protein denaturation suppression by enalapril, which is parallel to the standard drug piroxicam. Exposure to external stressors such as heat, organic solvents, strong acids and bases can lead to the denaturation of proteins, resulting in the disruption of their secondary and tertiary structures. The protein denaturation mechanism includes variations in disulfide, hydrogen, electrostatic and hydrophobic bonding [22]. It has been observed that in RA pathophysiology, protein denaturation can lead to auto-antigen production that can result in adverse outcomes in cartilage and bone deterioration [34]. Thereby, we can conclude from our results that enalapril might exert its anti-arthritis attribute by remarkably inhibiting protein denaturation.

Previous studies have shown that the erythrocytic membrane bears resemblance to the lysosomal membrane. Therefore, the stability of the erythrocytic membrane can be linked to the integrity of the lysosomal membrane. When RBCs are exposed to a hypotonic solution, membrane lysis occurs [24]. Thus, suppression of RBCs' haemolysis in hypotonic media provides a mechanism for evaluating lysosomal membrane integrity. During the inflammatory process, the lysosomal membrane is ruptured, leading to the release of lysosomal components such as proteases, bacterial enzymes and phospholipase A2, which causes the breakdown of phospholipids with the resultant release of inflammatory mediators [35]. Thus, if lysosomal membrane rupturing can be prevented, there will be no release of lysosomal content that can provoke inflammation. As inflammation has been described as the hallmark of arthritis, preventing inflammation can be a useful strategy for managing arthritis. In the current investigation, enalapril displayed concentration-dependent stabilisation of RBCs exposed to hypotonic media. This protective attribute of enalapril can be delineated as an indicator of its anti-arthritis potential.

Formaldehyde-induced arthritis has been described as one of the best methods for assessing anti-proliferative and anti-arthritis activity due to its similarity to human arthritis [36]. One of the finest methods for assessing anti-proliferative activity and screening anti-arthritis agents is the inhibition of formaldehyde-induced oedema. A localised inflammatory response to formaldehyde occurs in two stages. Initially, substance P is released (neurogenic phase), while histamine, serotonin, bradykinin and prostaglandins are released in the late phase (inflammatory phase), resulting in substantial vasodilation and permeability [37]. It has been stated that medications with a central mechanism of action consistently inhibit both phases, while those with a peripheral mechanism of action inhibit the late phase. In the current investigation, enalapril significantly inhibited formaldehyde-induced oedema during both the neurogenic and inflammatory

phases. Thus, it can be established that enalapril might exert its anti-arthritic potential by acting centrally. The immunosuppressive effects of enalapril were evaluated using myelosuppression brought on by cyclophosphamide and DTH brought on by DNCB. DNCB administration to the skin promotes an influx of macrophages at the injected site, causing the release of IL-12 and IL-18 [38]. The differentiation of Th1 cells is then brought on by these cytokines. These variables cause the skin at the reaction site to become thicker, which is a common sign of DTH [28]. In contrast to the group receiving enalapril, the positive control group showed a more profound inflammatory response to the allergen. Moreover, a dose-dependent decrease in skin thickness in the enalapril-treated groups was observed as compared to the positive control group. DTH, a T-cell-mediated response, is regulated by a range of prostaglandins, including PGE₂, PGI₂ and PGD₂, as well as by T-cell-specific cytokines. Consequently, DTH inhibition can also aid in determining the potential of enalapril in decreasing prostaglandin and cytokine production, which can contribute to its anti-arthritic potential as well.

As mentioned above, the cyclophosphamide-induced myelosuppression assay was used to assess the immunomodulatory effects of enalapril on cell-mediated immunity. Cyclophosphamide suppresses bone marrow by alkylating DNA [39]. Enalapril's effects were examined in both healthy and neutropenic mice that had been subjected to cyclophosphamide. Enalapril had an immunosuppressive impact by lowering total leukocyte counts (TLCs) and differential leukocyte counts in a dose-dependent manner (DLCs). The WBCs are a key component of the immune system. They are known to recognise the pathogen and assist in the immunological response; therefore, an increase or decrease in WBCs will have a direct impact on the immune system [40]. By phagocytosing the pathogens, WBCs combat the infection-producing agents and deliver them to phagosomes, where they are destroyed. Lymphocytes are regarded as an essential component of the immune system because they promote the development and proliferation of T-cells and B-cells, which are crucial for controlling immunity. Thus, enalapril might aid in the management of autoimmune disorders through possible suppression of the immune system.

Conclusions

In light of the aforementioned analysis, it is plausible that enalapril has been found to significantly inhibit immune function and have anti-arthritic effects in experimental studies. Enalapril significantly reduced formaldehyde-induced paw oedema, stabilised the RBC membrane and prevented protein denaturation. Enalapril's capacity to lower DTH brought on by DNCB

and all haematological parameters in cyclophosphamide-induced myelosuppression are additional indications of its immunosuppressive qualities. Enalapril can therefore be suggested as a potential treatment for arthritic symptoms, but more thorough mechanistic research is needed to pinpoint the drug's precise mechanism of action.

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Conflict of interest

The authors declare no conflict of interest.

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