



OPEN Multi-target drug discovery for rheumatoid arthritis: a comprehensive computational approach using bioactive compounds

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Rheumatoid arthritis (RA) is one of the most common inflammatory diseases in the world. Due to this importance, several drugs have been produced against effective targets in the pathogenesis of the disease. Considering various inflammatory pathways involved in RA, targeting each of these pathways alone cannot achieve sufficient improvement of the patients. Therefore, this study aimed to introduce safe natural product drug candidates screened in silico against several important protein targets (multi-targets) in the pathogenesis of RA. Tyrosine kinase 2 is a member of the Janus Kinase family, and due to its role in the signaling of numerous cytokines, its inhibition is considered an effective treatment option in inflammatory diseases. IL-6 is one of the most important innate immune cytokines that is secreted from activated macrophages in RA, and its level is directly related to the severity of joint destruction. Therefore, its inhibition plays an important role in improving the symptoms of RA. Also, the severity of the disease is directly related to the level of ACPA in the serum and synovial fluid of patients. Therefore, inhibiting its production by precluding the differentiation of B lymphocytes into plasma cells can improve the disease. In this study, after Virtual Screening, Molecular Docking, and Molecular Dynamics, we introduced Rutaecarpine, Hecogenin, Angustine, and Vomicine as new drug candidates for the treatment of RA. Rutaecarpine, Hecogenin, and Angustine inhibit all three targets with high affinity and stability, while Vomicine inhibits both TYK2 and IL-6 but not CD20. Future experimental studies can verify these findings.

Keywords Rheumatoid arthritis, MD simulation, Bioactive compounds, Anti-inflammation, Multi-target drugs

Abbreviations

RA	Rheumatoid Arthritis
DMARDs	disease-modifying antirheumatic drugs
TYK2	Tyrosine kinase 2
IL-6	Interl
JAK	Janus kinase
IL	interleukin
INF-I	type I interferons
Th	T helper
AD	atopic dermatitis
TLS	Tumor Lysis Syndrome
PDB	Protein Data Bank

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ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
MD	Molecular dynamics
PME	particle mesh Ewald
ΔG_{bind}	binding free energy
ΔG_{RL}	free energy of the complex
ΔG_{R}	free energy of the receptor
ΔG_{L}	free energy of the ligand
ΔE_{MM}	molecular mechanical energy in the gas phase
ΔE_{vdW}	van der Waals interaction energy
ΔE_{ele}	electrostatic energy
ΔG_{sol}	solvation free energy
CNS	central nervous system
Rg	radius of gyration
RDF	radial distribution function
EMS	Er Miao San
LPS	lipopolysaccharide

Rheumatoid arthritis (RA) is one of the most common autoimmune inflammatory diseases in the world. Between 0.5 and 1% of the world's population is affected by this disease¹. Genetic factors play a significant role (50–60%) in developing rheumatoid arthritis². In addition to genetic factors, environmental factors also play a role in the development of RA. The characteristic feature of this disease is joint inflammation, which can manifest in the form of joint synovitis³ and progressive destruction of bones and cartilage in multiple joints⁴. One of the most common clinical manifestations of this disease is osteoporosis, which is caused by inflammation and the chronic use of certain drugs such as glucocorticoids⁵. The first line of treatment for RA is corticosteroids, which have many side effects, including metabolic⁶, cardiovascular^{7,8}, gastrointestinal⁹ and osteoporosis¹⁰ problems. Another common category of drugs is disease-modifying antirheumatic drugs (DMARDs), which can lead to cytopenias¹¹, liver damage¹², and gastrointestinal problems¹³. Therefore, finding efficient drugs with new therapeutic targets with fewer side effects is significant.

Tyrosine kinase 2 (TYK2) is a member of the Janus kinase (JAK) family, which plays a role in the signaling pathway of interleukin (IL) 12, IL-23, and type I interferons (IFN-I)¹⁴. IL-12 produced by macrophages plays a key role in promoting the polarization of naive T cells to Thelper 1¹⁵. For this reason, inhibition of the IL-12 signaling pathway is important in RA disease. Another cytokine identified in the serum and synovial fluid is IL-23, associated with increased disease severity in RA patients¹⁶. IL-23 is secreted from activated macrophages during the disease. It causes the differentiation and survival of T helper (Th)17, and plays an important role in inflammatory diseases, especially RA, due to the secretion of IL-17, IL-1, TGF- β , and TNF- α cytokines^{17–20}. Due to the importance of TYK2 in inflammatory cytokine signaling, TYK2 inhibitors have been used in several inflammatory diseases such as psoriasis, lupus, Crohn's, psoriatic arthritis, and ulcerative colitis. Brepocitinib is one of the TYK2 inhibitors produced by Pfizer and is in clinical trials for atopic dermatitis (AD). Sotykto (Deucravacitinib) is one of the most effective selective TYK2 inhibitors of the BMS pharmaceutical company.

Activated macrophages in the synovium of RA patients are another important cell in the pathogenesis of RA disease²¹, which by producing inflammatory cytokines cause damage and erosion of joints, destruction of cartilage, and progression of the disease towards chronic inflammation²¹. IL-6 is one of the most important innate immune cytokines that is secreted from activated macrophages in RA, and its level is directly related to the severity of joint destruction²². The important role of IL-6 in the pathogenesis of RA has made this cytokine an effective therapeutic target. To play its role, IL-6 requires binding to the IL-6 alpha receptor and gp130 to form a hexamer structure²³. The compounds introduced today to inhibit the function of IL-6 prevent the formation of this hexamer structure by targeting the binding region of IL-6 to IL-6 alpha receptor (23). In this study, this region has been considered as the target of the compounds.

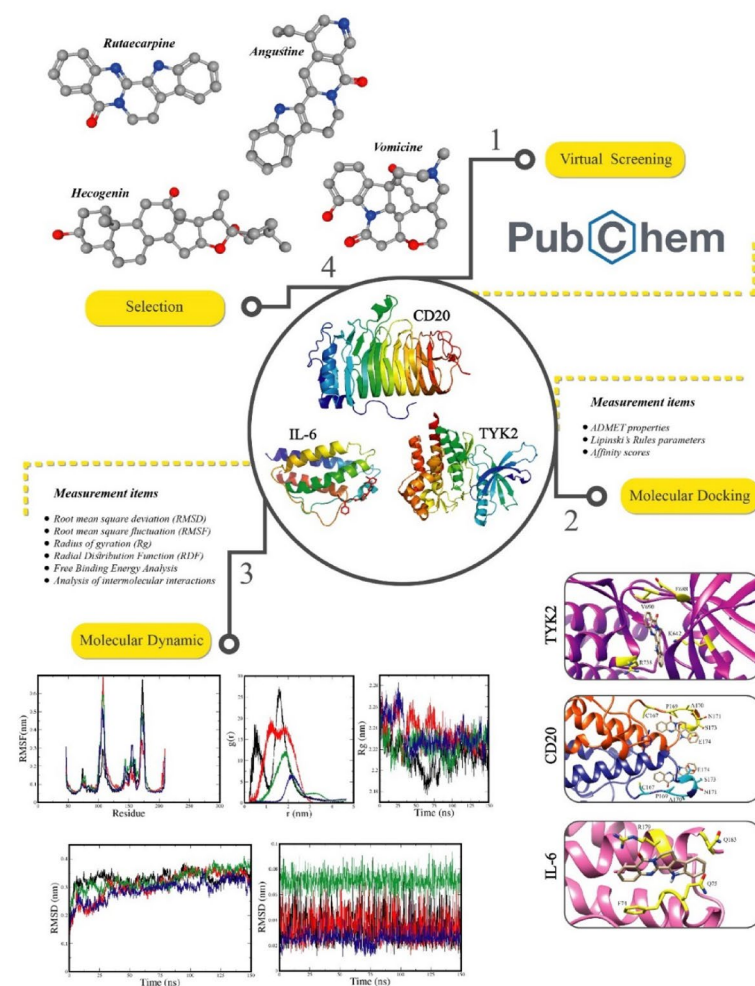
In addition to T lymphocytes and macrophages, B lymphocytes can differentiate into plasma cells by presenting citrullinated antigens to Th1 and produce antibodies against citrullinated proteins in the pathogenesis of RA, which play an important role in this regard^{24,25}. So the severity of the disease has a direct relationship with the level of ACPA in the serum and synovial fluid of the patients, and even a few years before the RA initiation, the level of this antibody in the body starts to rise^{24,26,27}. One of the B lymphocyte surface markers that plays a role in the development and differentiation of B lymphocytes into plasma cells is CD20²⁸. So far, several studies have investigated the effect of CD20 inhibition in improving the symptoms of RA^{28,29}. Rituximab is an anti-CD20 monoclonal antibody that showed remarkable results in the phase III clinical trial, so today, big companies worldwide have started producing Rituximab biosimilars³⁰. In spite of the acceptable effectiveness of Rituximab, considerable side effects have been reported. Tumor Lysis Syndrome (TLS)³¹, serious infections, heart and kidney complications, and gastrointestinal problems are some of the common side effects of Rituximab^{32–35}. The binding site of Rituximab to CD20 has been selected as the target of compounds in this study.

The simultaneous targeting of IL-6, TYK2, and CD20 provides an RA therapy that is aimed at the complex immunopathology of the disease. The multi-target approach is predicted to have synergistic effects by simultaneously blocking proinflammatory cytokine activity, immune cell signaling, and autoreactive B cell depletion, with the possibility of having improved outcomes in patients who do not respond to single-target medications. The latest requirement for combination therapies is also supported by the literature on dual-target agents, such as a publication demonstrating the efficacy of a dual-target-directed agent against IL-6 receptor and TNF α in experimental arthritis, suggesting that more than one pathway can be targeted to enhance therapeutic effects³⁶. While there is no specific study for combination therapy on IL-6, TYK2, and CD20, the function of

each alone and the success of JAK inhibitors (which act on TYK2) in combination with B-cell depleting therapies provide a strong rationale for this strategy.

The use of natural compounds from the past has played an important role in the discovery and development of drugs for various diseases, such as cancer and infectious diseases³⁷. According to published statistics, the global herbal medicine market was valued at \$151.91 billion in 2021 and is expected to reach \$347.5 billion by 2029. The World Health Organization has announced that the trade of medicinal plants, herbal raw materials, and herbal medicines is increasing with an annual growth rate of 15%, which highlights the importance of medicinal plants as the main source for medicinal compounds³⁸.

In various studies, the therapeutic effect of different herbal compounds in improving the symptoms of RA has been investigated. The great advantage of drugs that are made based on natural compounds is their low side effects compared to synthetic drugs. Relevantly, it should be noted that due to the increasing trend of diseases throughout the world, the need to discover and develop novel drugs with greater effectiveness seems essential. The process of drug discovery and development using conventional approaches takes about 10–14 years, and is a very risky and costly process³⁹. By using computer-aided drug design methods, we can reduce the cost of drug discovery and development by 50%⁴⁰. In silico methods include target mining, virtual screening of different chemical compounds to find effective drug candidates, and evaluation of their potential toxicity. Therefore, this approach reduces the number of chemical compounds that must be experimentally evaluated, and at the same time increases the success by removing ineffective and toxic compounds⁴¹. Studies have provided examples of in-silico approaches applied to optimize protein-ligand interactions and predict pharmacokinetic properties^{42,43}. Emerging algorithms allowing for integrations of multi-omics data have also extended the boundaries of in-silico methodologies, permitting more comprehensive simulations of disease mechanisms⁴⁴. In this study, after virtual screening on 2299 compounds, we checked the Physicochemical characteristics, ADMET properties, and Affinity scores of different compounds in complex with CD20, TYK2, and IL-6. Then we select the best compounds, and after Molecular Dynamics, we analyze the binding free energy and intermolecular interactions of complexes.



Methods

Structure-Based drug design using virtual screening

The wild types of human CD20, TYK2, and IL-6 have been selected with 210, 317, and 299 amino acid lengths, and the crystal structures (PDB ID: 6VJA, 6NZP, and 1P9M, respectively) and retrieved from the Protein Data Bank (PDB)^{23,28,45}. AutoDock FR software was applied to prepare proteins for virtual screening and molecular docking⁴⁶. The ligands (herbal bioactive compounds) were processed by the OpenBabel program to add hydrogen (H) and generate of 3D structure in PDB format. The selected grid box size and binding sites are shown in Table 1. The AutoDock Vina tool⁴⁷ was employed to carry out docking studies using the PDBQT files of CD20, TYK2, and IL-6 structures and ligand library (a total of 2299 molecules). The evaluation of herbal bioactive compounds' properties about Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) and Lipinski's Rules was conducted using the OSIRIS Property Explorer and pkCSM websites^{48,49}. Each docked complex 3D structure was analyzed by applying the PyMOL visualization tool^{50,51}.

Physicochemical characteristics and ADMET properties

In this study, the OSIRIS Property Explorer and pkCSM platforms were employed to assess a range of parameters for chosen candidates. These parameters encompass various physicochemical characteristics, including the number of rotatable bonds, octanol-water partition coefficient, molecular weight, water solubility, hydrogen bond donors and acceptors, and topological polar surface area. Additionally, pharmacokinetic parameters such as Caco-2 permeability, P-glycoprotein inhibition, and human intestinal absorption were determined, along with distribution metrics like central nervous system permeability, volume of distribution, and blood-brain barrier penetration. In addition, metabolic parameters were analyzed, including the potential to inhibit cytochrome P450 subtypes. Excretion factors such as total clearance and renal OCT2 substrate potential, as well as toxicity indicators like AMES toxicity, inhibition of human ether-related gene channels, acute and chronic toxicity in oral rats, and skin sensitization, were also examined.

Molecular dynamics (MD) simulations

Molecular dynamics (MD) simulations were conducted on the potentially docked complexes using GROMACS-2022 to analyze the behavior of the ligand-receptors in physiologic conditions^{52,53}. The protein's topology was generated with the OPLS-AA force field⁵⁴. Ligand molecular topology files were generated using the LigParGen server (<https://traken.chem.yale.edu/ligpargen/>). All systems were solubilized in a cubic box using the TIP3P water model⁵⁵, and the systems were neutralized by adding counter ions (Na⁺ and Cl⁻) at a concentration of 0.15 M. The energy of the neutralized systems was minimized through the steepest descent and conjugate gradient methods, with 50,000 steps for each. System equilibration involved performing volume (NVT) regulation and pressure (NPT). The temperature was gradually raised to 310 K in the NVT ensemble, followed by pressure and density equilibration in the NPT ensemble. The main simulation runs were performed for 150 ns for each complex system. The SHAKE algorithm was employed to restrain hydrogen atoms at their equilibrium distances. Periodic boundary conditions were applied throughout the MD simulations, and long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method⁵⁶. Coulombic and van der Waals interactions were cut off at 1.2 nm, and bond lengths and angles were constrained with the LINCS algorithm⁵⁷. The MD trajectory data were recorded at intervals of 10 ps. To model the membrane protein CD20, we used the CHARMM-GUI web server to create a membrane bilayer and insert the protein ion channel through a replacement method. The CHARMM36 force field was employed for this system. The bilayer was made up of 512 molecules of POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine), and we set the water layer thickness on each side of the membrane to 2.2 nm. After setting up the model, we conducted equilibration phases, applying position restraints on the protein, lipids, and drug molecules for 1 ns using the NVT and NPT ensembles through standard coupling techniques. Finally, we ran the production phase for 150 ns without any restraints on the molecules. Hydrogen bond (H-bond), Root Mean Square Fluctuation (RMSF), Root Mean Square Deviation (RMSD), Radius of gyration (Rg), and Radial Distribution Function (RDF) analysis were conducted using the GROMACS tool gmx hbond, gmx rmsf, gmx rms, gmx gyrate, and gmx rdf, respectively.

Protein	Grid box size	Binding site
CD20 (6VJA)	18.750 Å×14.250Å×30.250Å	C167 P169 A170 N171 S173 E174
TYK2 (6NZP)	18.750 Å×16.500Å×18.750Å	K642 E688 V690 R738
IL-6 (1P9M)	32.250Å×21.000Å×39.750Å	F74 Q75 R179 Q183

Table 1. Grid box size surrounding binding site of CD20, TYK2, and IL-6.

Clustering analysis

We conducted a clustering analysis on the bound compounds to uncover differences in their binding poses. For each compound, we identified prominent clusters and selected the central structure to serve as the representative. Furthermore, we analyzed molecular dynamics (MD) simulation trajectories utilizing GROMACS with the GROMOS method, applying an RMSD cutoff of 0.2 nm. To reduce noise, we aligned the trajectories with the protein backbone and concentrated on the residues at the binding site for clustering. This methodology enables us to study key interactions between ligands and proteins effectively.

Binding free energy

To analyze the energy components that contribute to the binding of proteins with ligands, the MM-PBSA method was employed, as implemented in the *g_mmpbsa* program (solute = 2, solvent = 80, grid spacing = 0.5 Å, and 100 snapshots). This approach calculates the binding free energy (ΔG_{bind}) of the receptor-ligand complex by considering the difference between the free energy of the complex (ΔG_{RL}) and the free energies of the individual components (ΔG_{R} for the receptor and ΔG_{L} for the ligand). Mathematically, this relationship is expressed as:

$$\Delta G_{\text{bind}} = \Delta G_{\text{RL}} - \Delta G_{\text{R}} - \Delta G_{\text{L}} \quad (1)$$

The free energies are derived from multiple energy components, as described in the following equations:

$$\Delta G = \Delta E_{\text{MM}} + \Delta G_{\text{sol}} \quad (2)$$

Here, the molecular mechanical energy in the gas phase (ΔE_{MM}) is calculated by summing the van der Waals interaction energy (ΔE_{vdW}) and the electrostatic energy (ΔE_{ele}):

$$\Delta E_{\text{MM}} = \Delta E_{\text{vdW}} + \Delta E_{\text{ele}} \quad (3)$$

Moreover, the solvation free energy (ΔG_{sol}) is further divided into polar ($\Delta G_{\text{sol-pol}}$) and non-polar ($\Delta G_{\text{sol-np}}$) contributions:

$$\Delta G_{\text{sol}} = G_{\text{sol-pol}} + G_{\text{sol-np}} \quad (4)$$

This methodology allows for a comprehensive exploration of the energetic dynamics involved in receptor-ligand interactions⁵⁸.

Software tools

The softwares we used for virtual screening and MD simulation was The PyMOL Molecular Graphics System, Version 3.0, Schrödinger, AutoDock Vina, LigPlot + v.2.2, GROMACS-2022, AutoGridFR (AGFR).

Results

ADMET analysis and toxicity profiling

From a total of 2,299 herbal compounds obtained from the PubChem database, Rutaecarpine, Hecogenin, Angustine, and Vomicine were chosen based on their ADMET properties, Lipinski's Rules parameters, and affinity scores (Table 2). Generally, a threshold of > 30% for intestinal absorption and a Caco-2 cell permeability greater than 0.9 is considered acceptable. Additionally, these compounds exhibit a longer half-life in the human body, attributed to their low renal clearance rates. The permeability values for Rutaecarpine, Hecogenin, Angustine, and Vomicine are 1.26, 1.432, 1.282, −0.134, respectively, and with an intestinal absorption value of 97.294, 96.696, 98.509, and 98.304% (Table 3). Their permeability across the blood-brain barrier is recorded at 0.669, −4.043, 0.401, and 0.147, respectively, and also central nervous system (CNS) permeability is noted as −1.795, −1.443, −1.703, and −2.389, respectively, suggesting limited access to the CNS (Table 3). No toxicity or mutagenicity was detected (Table 3). They were subsequently selected for MD simulation. The Vina score and the 2D chemical structure of these compounds are presented in Table 2.

We have indeed performed comparisons between active compounds and inactive compounds like D-glucose, D-fructose, and Lactose (Table 2). The results demonstrated that active compounds bind to the binding site with higher affinity and effectively inhibit CD20, TYK2, and IL6 proteins. These comparisons revealed that the binding of active compounds to binding sites is specific and significant, rather than resulting from non-specific binding. The results indicate that our active compounds can be considered as potential drug candidates for further investigation.

Also, we have performed a validation of our docking protocol by re-docking the co-crystallized ligand from the TYK2–Deucravacitinib complex. The resulting root mean square deviation (RMSD) between the re-docked and crystallographic poses was 0.026 nm, indicating a high degree of accuracy in our docking procedure (Fig. 5S1).

Complex stability and compactness

The RMSD of the CD20 systems protein exhibited a similar pattern along the simulation time (Fig. 1A). Accordingly, in all the complexes, the CD20 protein RMSD increased sharply between 0.2 and 0.33 nm, and then the fluctuation rate decreased and reached a smooth plateau until the end of the simulation. Therefore, CD20 had acceptable stability for extra analyses (Table 4). As depicted, the CD20 mean RMSD in complex with Rutaecarpine, Hecogenin, Angustine, and Vomicine included 0.33, 0.32, 0.31, and 0.29 nm, respectively. For deciphering the local fluctuations for each of the amino acids of the CD20 protein affected by bioactive compounds, the RMSF parameter was calculated (Fig. 1B). The mean RMSF values for Rutaecarpine, Hecogenin,

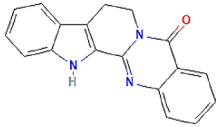
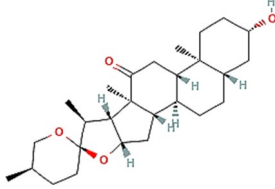
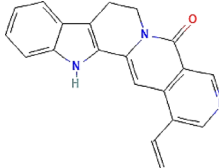
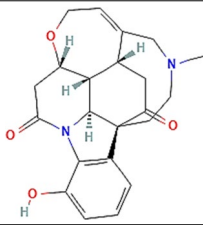
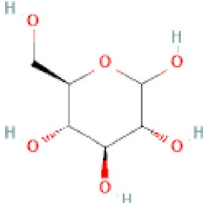
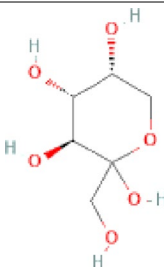
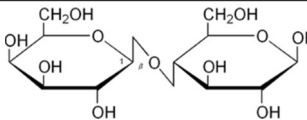
	Ligand Structure	CD20	TYK2	IL-6
Rutaecarpine (65752)		-7.3	-10.2	-7.0
Hecogenin (91453)		-7.2	-7.2	-7.3
Angustine (441983)		-7.4	-10.0	-7.1
Vomicine (101595)		-7.5	-8.7	-7.4
D-glucose		-4.3	-5.4	-4.0
D-Fructose		-4.1	-5.4	-4.2
Lactose		-4.1	-5.2	-4.1

Table 2. Affinity of candidate compounds and inactive compounds in complex with CD20, TYK2, and IL6.

Angustine, and Vomicine-bound CD20 were equal to 0.12, 0.14, 0.14, and 0.12, respectively, which highlighted the low local fluctuations in the complex systems (Table 4).

In addition, the radius of gyration (Rg) is an important measure that helps us understand the shape and compactness of a protein's conformation. In this study, we used Rg to assess the protein–compound complexes, as illustrated in Fig. 1C. There were higher Rg values, indicating an unstable structural conformation. On average, the Rg value for both the protein in complex with Rutaecarpine, Hecogenin, Angustine, and Vomicine was 2.21, 2.22, 2.26, and 2.23 nm, respectively (Table 4). Rg analysis demonstrated higher values for all complexes at each initial time of simulation, but decreased until the end of simulation time with a similar final pattern. Overall, minimal changes during the simulations occurred, suggesting that the protein maintained its structural stability and experienced acceptable compactness (Fig. 2C).

To gain insights into how the compound is distributed within the protein binding site and its behavior during our simulations, we examined the radial distribution function (RDF). The bin width used for RDF in all

	Rutaecarpine (65752)	Hecogenin (91453)	Angustine (441983)	Vomicine (101595)
Water solubility	-3.459	-5.028	-4.12	-2.427
TPSA	48.46	55.76	48.99	70.08
H bond acceptor	4	4	4	6
H bond donor	1	1	1	1
Nb rotatable bonds	0	0	1	0
Molecular weight	287.32	430.63	313.36	380.44
Caco-2 permeability	1.26	1.432	1.282	-0.134
P-glycoprotein I inhibitor	NO	Yes	Yes	No
P-glycoprotein II inhibitor	Yes	Yes	Yes	No
Intestinal absorption (human)	97.294	96.696	98.509	98.304
BBB permeability	0.669	-4.043	0.401	0.147
CNS permeability	-1.795	-1.443	-1.703	-2.389
Total Clearance	0.756	0.315	0.511	0.887
Renal OCT2 substrate	No	Yes	Yes	No
AMES toxicity	Yes	No	Yes	No
Oral Rat Acute Toxicity (LD50)	2.431	2.028	2.532	2.755
Oral Rat Chronic Toxicity (LOAEL)	0.709	1.133	0.245	1.225
Skin Sensitisation	No	No	No	No

Table 3. ADME analysis and toxicity profiling of candidate compounds obtained from OSIRIS property explorer and pkcsm.

complexes was 0.002 nm. This tool helps to identify patterns, with peaks indicating favored distances that show where ligands are likely to be situated about specific amino acids, specifically A170 in the binding site. A170 is one of the important residues at the binding site of CD20.

The results presented in Fig. 1D; Table 4 illustrate the variations in the RDF throughout the simulation. Notably, the compounds Rutaecarpine and Angustine showed the highest peaks at 1.8 nm and 1.4 nm, respectively, while Hecogenin and Vomicine had peaks around 2 nm from the reference residue A170. This data suggests that Rutaecarpine and Angustine had stability with minimal fluctuations, maintaining their conformations at their binding sites. On the other hand, the RDF for Hecogenin and Vomicine indicated greater mobility within the protein binding sites, suggesting interactions with non-specific residues, as shown in Fig. 1D.

For checking compounds' conformational changes at the binding site of CD20 protein, the mean RMSD values of the compounds' heavy atoms were measured as follows: 0.03, 0.07, 0.04, and 0.03, respectively, for Rutaecarpine, Hecogenin, Angustine, and Vomicine. These values suggest that the compounds mostly effectively interact with the binding site, maintaining proper conformations. This indicates a favorable stability of the compounds within the complex structures (Fig. 1E). The number of H bonds is shown in Fig. 1F.

The RMSD of TYK2 systems increased between 0.2 and 0.5 nm with the gradual decrease in oscillation ranges, and then a smooth plateau was observed until the end of the simulation (Fig. 2A). Therefore, these results confirm that TYK2 has good stability for further analyses (Table 5). The mean RMSD of TYK2 in complex with Rutaecarpine, Hecogenin, Angustine, and Vomicine was 0.55, 0.48, 0.38, and 0.39 nm, respectively. Moreover, the mean RMSF values for Rutaecarpine, Hecogenin, Angustine, and Vomicine-bound TYK2 included 0.13, 0.16, 0.14, and 0.14, respectively (Fig. 2B), which indicated the low local fluctuations in the complex systems (Table 5).

On average, the Rg value for both the protein in complex with Rutaecarpine, Hecogenin, Angustine, and Vomicine was 1.97, 2.01, 1.98, and 1.99 nm, respectively (Table 5). Rg analysis showed higher values for all complexes at each initial time of simulation, but decreased until the end of simulation time with a similar final pattern. Overall, minimal changes during the simulations occurred, suggesting that, similar to CD20, TYK2 maintained its structural stability and experienced acceptable compactness (Fig. 2C).

As shown in Fig. 2D, we had variations in the RDF throughout the simulation. Notably, the compounds Rutaecarpine, Angustine, and Hecogenin showed the highest peaks at 0.6 nm, while Vomicine had peaks around 0.7 nm from the reference residue V138 that is at the binding site of TYK2 and has a Hydrogen bond with ligands. This data suggests that Rutaecarpine, Angustine, and Hecogenin had stability with minimal fluctuations, maintaining their conformations at their binding sites. Nonetheless, the RDF for Vomicine indicated greater mobility within the protein binding sites, suggesting interactions with non-specific residues (Fig. 2D).

The mean RMSD values of the compounds' heavy atoms were measured as follows: 0.03, 0.05, 0.02, and 0.03, respectively, for Rutaecarpine, Hecogenin, Angustine, and Vomicine. These values suggest that the compounds mostly effectively interact with the binding site, maintaining proper conformations. All candidate compounds had stability within the complex structures (Fig. 2E). The number of H bonds is shown in Fig. 2F.

The RMSD of the IL-6 system's protein exhibited a similar pattern along the simulation time (Fig. 3A), which increased between 0.2 and 1 nm, and then the oscillation ranges were decreased and reached a smooth plateau until the end of the simulation in all systems. Therefore, IL-6 had acceptable stability for extra analyses (Table 6). IL-6 mean RMSD in complex with Rutaecarpine, Hecogenin, Angustine, and Vomicine included 0.29, 1.19,

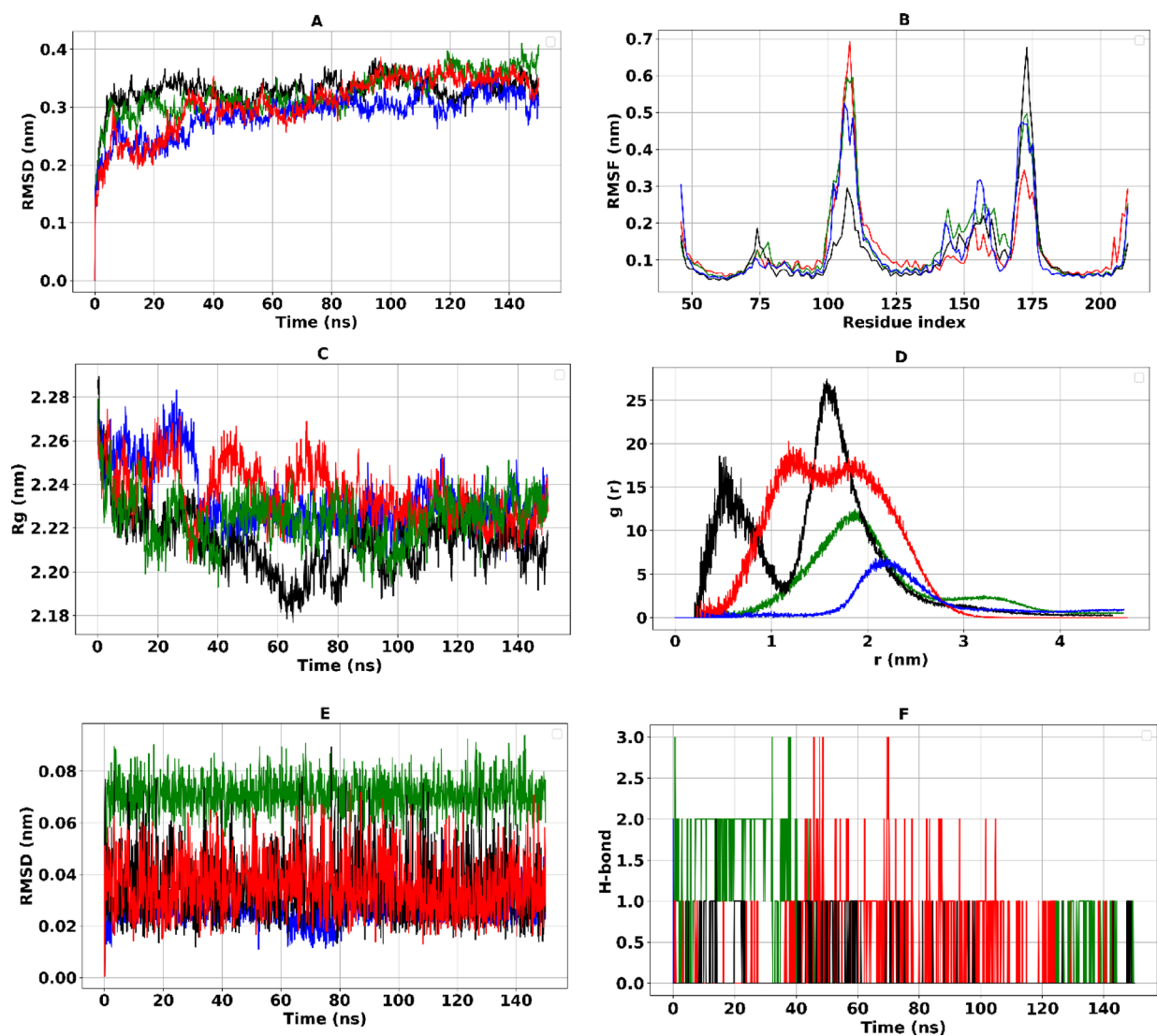


Fig. 1. Molecular dynamic results of CD20 complexes. The RMSD (A), and RMSF (B), of the Ca atoms. Radius of Gyration (Rg) (C). RDF (D). RMSD_{ligand} (E). H bond (F) The black, red, blue, and green colors are related to the CD20- Rutaecarpine, CD20- Angustine, CD20- Vomicine, and CD20-Hecogenin complexes, respectively.

	RMSF (Avg-nm)	RMSD (Avg-nm)	RMSD _{ligand} (Avg-nm)	RDF(Max)	Rg (Avg _z SD)
Rutaecarpine	0.12	0.33	0.03	27.44	2.21 _{±0.01}
Hecogenin	0.14	0.32	0.07	12.41	2.22 _{±0.01}
Angustine	0.14	0.31	0.04	20.27	2.26 _{±0.01}
Vomicine	0.12	0.29	0.03	6.89	2.23 _{±0.01}

Table 4. Molecular dynamic result of CD20 complexes.

0.74, and 0.84 nm, respectively. The mean RMSF values for Rutaecarpine, Hecogenin, Angustine, and Vomicine-bound IL-6 were equal to 0.13, 0.27, 0.22, and 0.19, respectively, which indicated the low local fluctuations in the complex systems (Fig. 3B) (Table 6).

On average, the Rg value for both the protein in complex with Rutaecarpine, Hecogenin, Angustine, and Vomicine was 1.64, 1.78, 1.75, and 1.77 nm, respectively (Table 6). In IL-6 complexes, like the two previously analyzed complexes, Rg analysis indicated higher values for all complexes at each initial time of simulation but decreased until the end of simulation time with a similar final pattern. Overall, minimal changes during the simulations occurred, suggesting that the protein maintained its structural stability and experienced acceptable compactness(Fig. 3C).

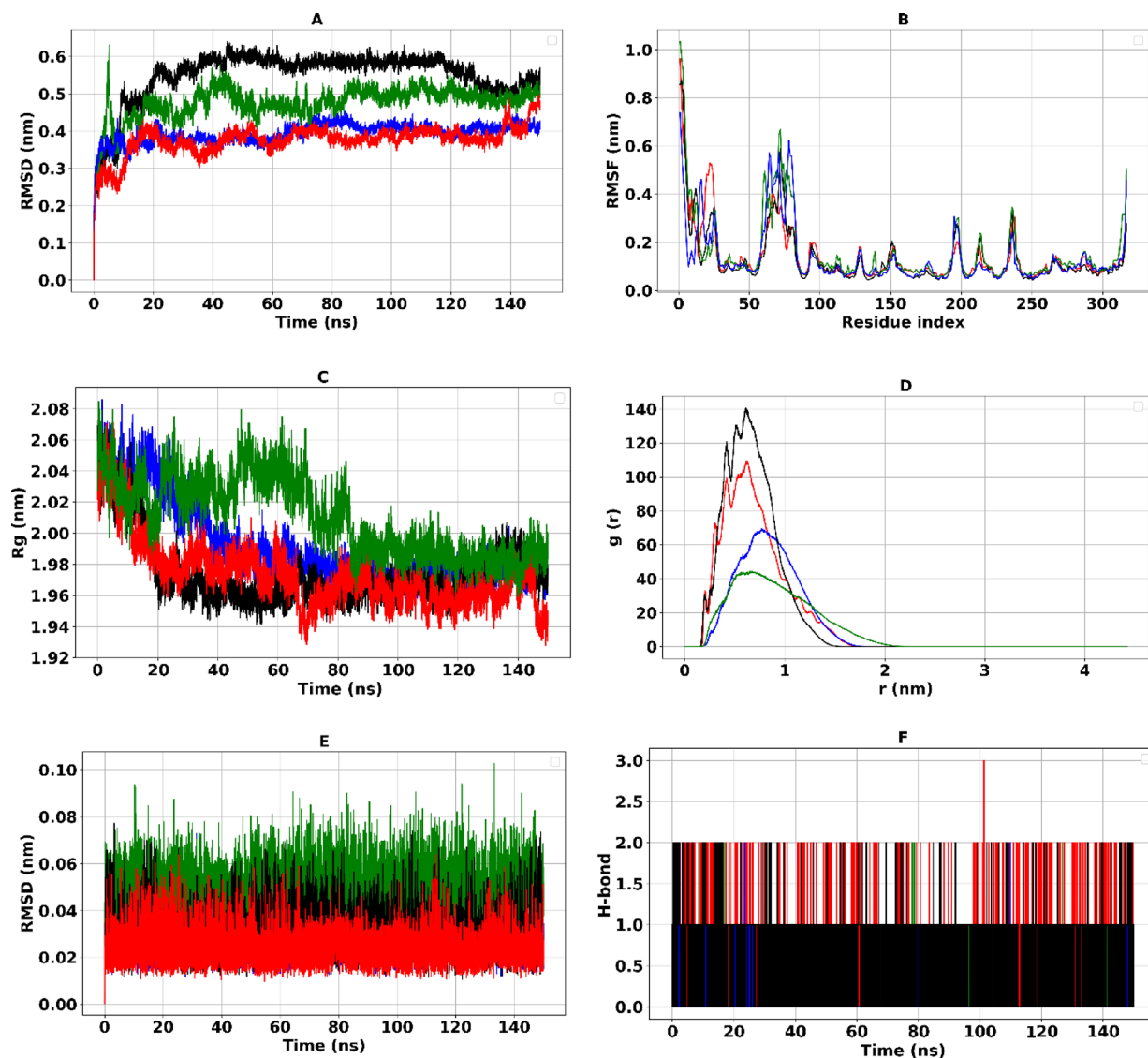


Fig. 2. Molecular dynamic results of TYK2 complexes. The RMSD (A), and RMSF (B), of the Ca atoms. Radius of Gyration (Rg) (C). RDF (D). RMSD_{ligand} (E). H bond (F). The black, red, blue, and green colors are related to the TYK2- Rutaecarpine, TYK2- Angustine, TYK2-Vomicine, and TYK2-Hecogenin complexes, respectively.

	RMSF (Avg-nm)	RMSD (Avg-nm)	RMSD _{ligand} (Avg-nm)	RDF(Max)	Rg (Avg _± SD)
Rutaecarpine	0.13	0.55	0.03	140.63	1.97 _± 0.02
Hecogenin	0.16	0.48	0.05	44.51	2.01 _± 0.03
Angustine	0.14	0.38	0.02	109.38	1.98 _± 0.02
Vomicine	0.14	0.39	0.03	69.36	1.99 _± 0.03

Table 5. Molecular dynamic result of TYK2 complexes.

The results presented in Fig. 3D; Table 6 illustrate the variations in the RDF throughout the simulation. Notably, the compounds Angustine, Vomicine, and Hecogenin showed the highest peaks at 0.5 nm, while Rutaecarpine had peaks around 0.4 nm from the reference residue R181, which is one of the important residues in the binding site of IL-6. This data suggests that Angustine, Vomicine, and Hecogenin had stability with minimal fluctuations, maintaining their conformations at their binding sites. On the other hand, the RDF for Rutaecarpine demonstrated greater mobility within the protein binding sites, suggesting interactions with non-specific residues, as shown in Fig. 3D.

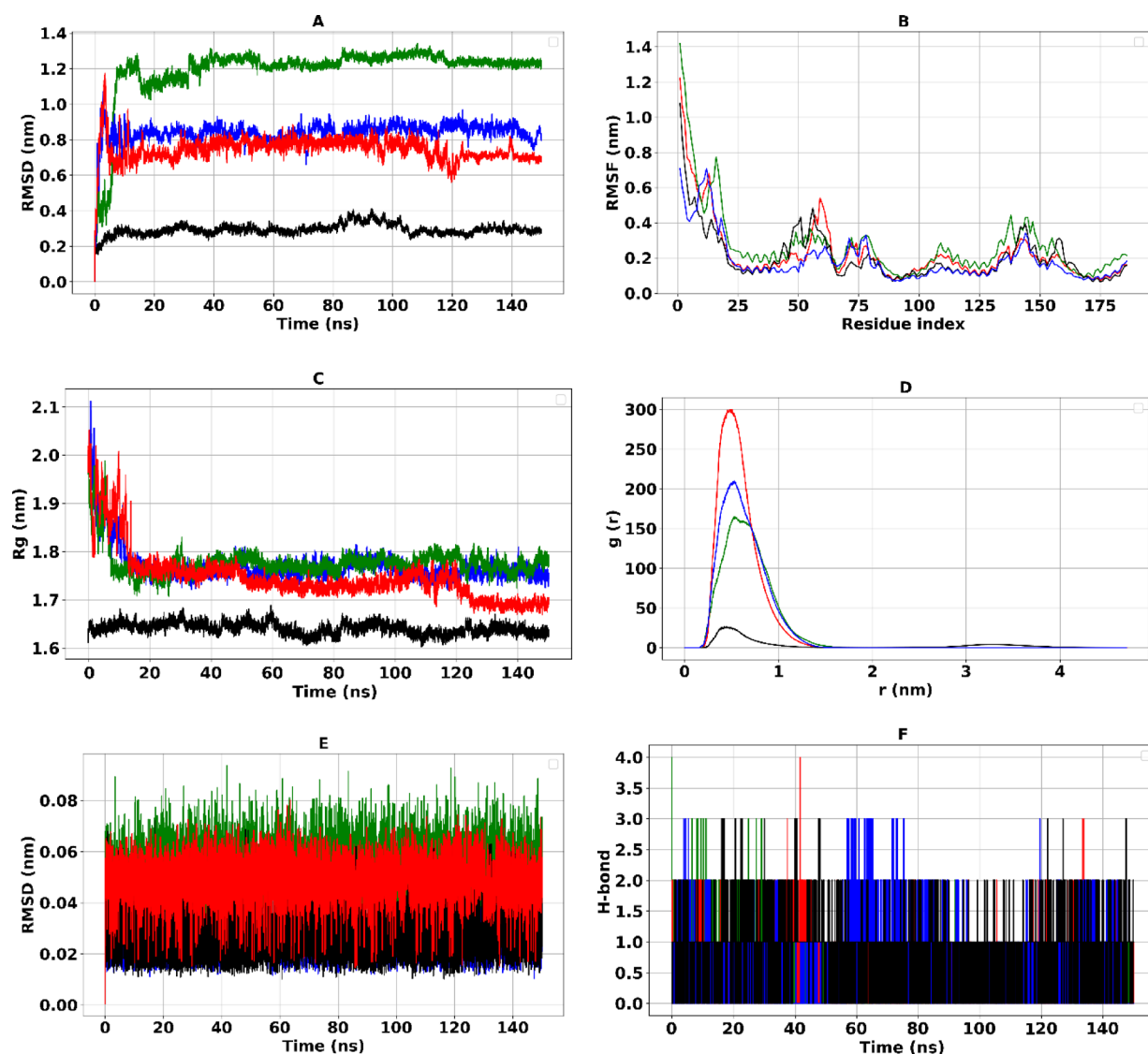


Fig. 3. Molecular dynamic results of IL6 complexes. The RMSD (A), and RMSF (B), of the Ca atoms. Radius of Gyration (Rg) (C). RDF (D). $\text{RMSD}_{\text{ligand}}$ (E). H bond (F). The black, red, blue, and green colors are related to the IL6- Rutaecarpine, IL6- Angustine, IL6- Vomicine, and IL6-Hecogenin complexes, respectively.

	RMSF (Avg-nm)	RMSD (Avg-nm)	$\text{RMSD}_{\text{ligand}}$ (Avg-nm)	RDF(Max)	Rg (Avg $\pm$ SD)
Rutaecarpine	0.13	0.29	0.03	26.59	1.64 $\pm$ 0.01
Hecogenin	0.27	1.19	0.05	164.52	1.78 $\pm$ 0.03
Angustine	0.22	0.74	0.05	299.75	1.75 $\pm$ 0.05
Vomicine	0.19	0.84	0.02	209.53	1.77 $\pm$ 0.04

Table 6. Molecular dynamic result of IL6 complexes.

For checking compounds' conformational changes at the binding site of IL-6 protein, the mean RMSD values of the compounds' heavy atoms were measured as follows: 0.03, 0.05, 0.05, and 0.02, respectively, for Rutaecarpine, Hecogenin, Angustine, and Vomicine. These values suggest that the compounds mostly effectively interact with the binding site, reaching proper conformations. This indicates a favorable stability of the compounds within the complex structures (Fig. 3E). The number of H bonds is shown in (Fig. 3F). In all our target complexes, specific residues whose RMSF exceeds 0.2 nm were in the loops, and they weren't binding-site elements (Fig. 4S1).

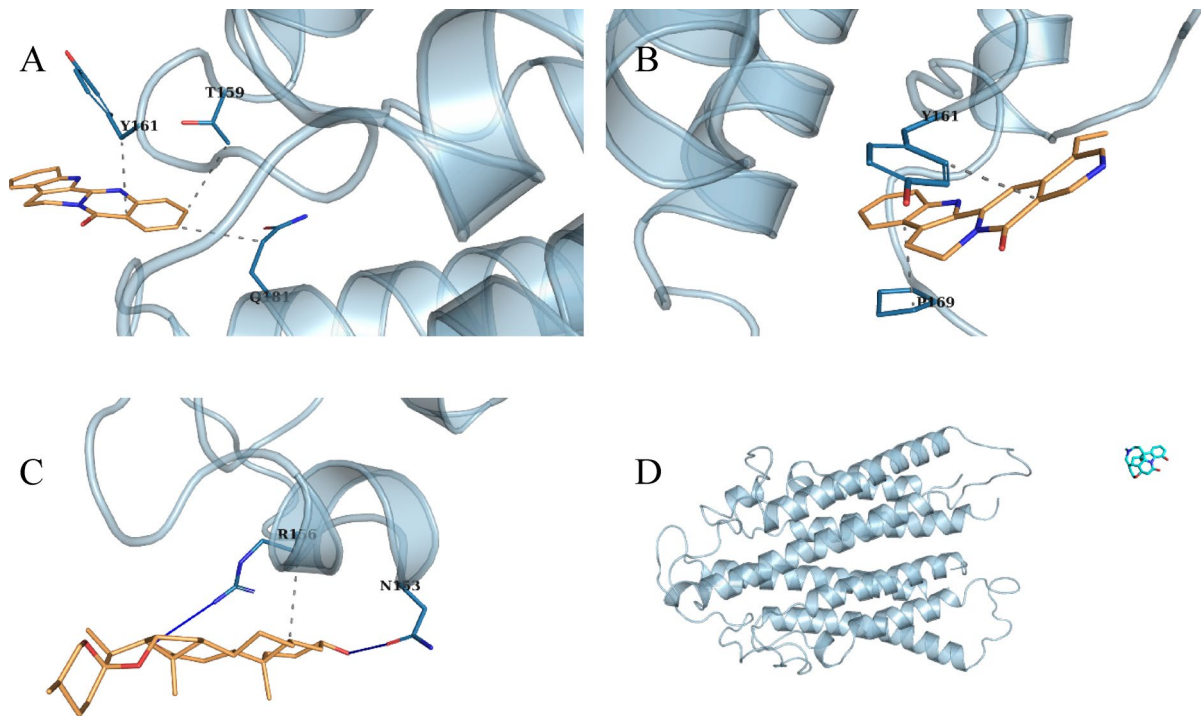


Fig. 4. CD20 complex with (A) Rutaecarpine, (B) Angustine, (C) Hecogenin, (D) Vomicine. The highlighted residues are marked with blue sticks. Blue lines indicate hydrogen bonds, gray dots represent hydrophobic interactions.

	Rutaecarpine	Hecogenin	Angustine	Vomicine
ΔE_{ele}	-9.73 ± 2.87	-0.37 ± 1.42	-4.95 ± 2.25	107.30 ± 14.56
ΔE_{vdW}	-60.36 ± 8.88	-75.62 ± 5.15	-119.48 ± 4.83	-0.03 ± 0.02
ΔG_{GB}	33.86 ± 11.78	50.78 ± 4.50	75.46 ± 3.07	52.93 ± 14.14
ΔG_{SA}	-7.85 ± 0.83	-10.03 ± 0.67	-11.38 ± 0.38	0.60 ± 0.21
$\Delta E_{non-polar}^a$	-68.21 ± 8.92	-85.65 ± 5.20	-130.85 ± 4.85	0.57 ± 0.21
ΔE_{polar}^b	24.14 ± 12.13	50.41 ± 4.72	70.52 ± 3.80	160.23 ± 20.30
ΔG_{bind}	-44.40 ± 1.89	-35.06 ± 4.36	-60.40 ± 3.54	160.36 ± 22.85

Table 7. Free binding energy and the individual energy contributions result from CD20 complexes (kJ/mol).

	Rutaecarpine	Hecogenin	Angustine	Vomicine
ΔE_{ele}	-17.49 ± 1.87	-0.15 ± 2.51	3.66 ± 2.65	-32.08 ± 5.60
ΔE_{vdW}	-146.06 ± 2.95	-124.91 ± 4.56	-167.41 ± 4.13	-123.67 ± 3.53
ΔG_{GB}	82.86 ± 3.57	47.98 ± 5.06	64.20 ± 3.22	104.21 ± 8.17
ΔG_{SA}	-15.86 ± 0.22	-18.28 ± 0.27	-17.76 ± 0.25	-15.25 ± 0.34
$\Delta E_{non-polar}^a$	-161.93 ± 2.96	-143.19 ± 4.57	-185.17 ± 4.14	-138.92 ± 3.55
ΔE_{polar}^b	65.37 ± 4.03	47.83 ± 5.64	67.86 ± 4.16	72.13 ± 9.90
ΔG_{bind}	-96.59 ± 4.78	-95.27 ± 3.72	-117.36 ± 5.73	-66.73 ± 3.18

Table 8. Free binding energy and the individual energy contributions result from TYK2 complexes (kJ/mol).

Free binding energy and interactions analysis

The binding free energy (ΔG_{bind}) values and van der Waals (ΔE_{vdw}) and electrostatic energy (ΔE_{ele}) components for CD20, TYK2, and IL-6 complexes during MD simulation are in Tables 7, 8 and 9. The MMPBSA method was used to assess how each compound interacts with the binding landscape energetically and determine the binding free energy. The computed binding free energy between Angustine and CD20 was -60.40 ± 3.54 kJ/mol (Table 7). It was better than other candidate compounds in complex with CD20. This complex exhibited

	Rutaecarpine	Hecogenin	Angustine	Vomicine
ΔE_{ele}	-16.48 ± 2.39	-19.41 ± 1.33	-12.91 ± 3.43	-19.06 ± 3.37
ΔE_{vdW}	-149.89 ± 3.44	-154.57 ± 6.17	-147.81 ± 6.39	-119.35 ± 5.56
ΔG_{GB}	64.29 ± 1.88	70.66 ± 1.05	73.56 ± 6.89	72.13 ± 4.64
ΔG_{SA}	-15.27 ± 0.19	-15.35 ± 0.32	-15.06 ± 0.37	-13.34 ± 0.69
$\Delta E_{non-polar}^a$	-165.16 ± 1.81	-169.92 ± 6.17	-162.87 ± 4.40	-132.69 ± 5.60
ΔE_{polar}^b	47.81 ± 3.04	51.25 ± 1.69	60.65 ± 7.69	53.07 ± 5.73
ΔG_{bind}	-117.53 ± 2.63	-118.74 ± 5.61	-102.19 ± 5.58	-79.77 ± 6.19

Table 9. Free binding energy and the individual energy contributions result from IL6 complexes (kJ/mol). a $\Delta E_{nonpolar} = \Delta G_{sol-np} + \Delta E_{vdw}$. b $\Delta E_{polar} = \Delta G_{sol-pol} + \Delta E_{ele}$.

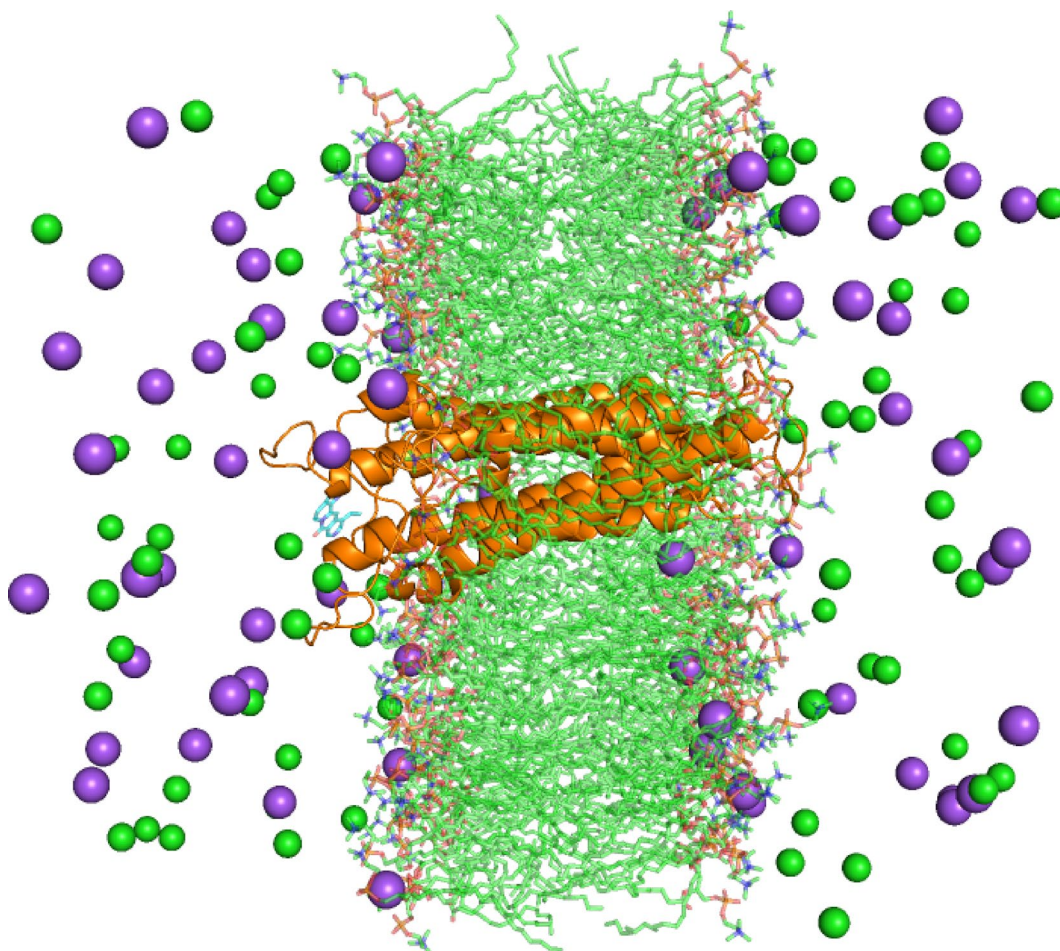


Fig. 5. Vomicine in complex with CD20 in the cell membrane. Vomicine is presented with blue. CD20 is presented with orange.

two hydrophobic interactions (Fig. 4B) (Fig. 1B S1). Although CD20 in complex with Rutaecarpine had three hydrophobic interactions (Fig. 4A) (Fig. 1A S1), ΔG_{bind} was -44.40 ± 1.89 kJ/mol (Table 7). Also, CD20 in the complex with Hecogenin had one hydrophobic interaction and two H-bonds (Fig. 4C) (Fig. 1C S1), and ΔG_{bind} was -35.06 ± 4.36 kJ/mol (Table 7). In all of these complexes, Non-polar interactions had the main effect on ΔG_{bind} of these complexes. But ΔG_{bind} of Vomicine in complex with CD20 was 160.36 ± 22.85 kJ/mol, which shows that it could not strongly bind to the binding site on CD20 and it was separated after 150 ns MD simulation (Fig. 4D; Table 7) (Fig. 1D S1). Therefore, it can be claimed that it is not a potential inhibitor of the CD20 (Fig. 5).

Similar to CD20 complexes, Angustine was the best compound for inhibition of TYK2. ΔG_{bind} of Angustine in complex with TYK2 was -117.36 ± 5.73 kJ/mol (Table 8) and had seven hydrophobic interactions and one H-bond (Fig. 6B) (Fig. 2B S1). Rutaecarpine and Hecogenin had similar ΔG_{bind} values, -96.59 ± 4.78 kJ/mol and -95.27 ± 3.72 kJ/mol, respectively (Table 8). Rutaecarpine in complex with TYK2 had five hydrophobic interactions and one H-bond (Fig. 6A) (Fig. 2A S1). Hecogenin in complex with TYK2 had two hydrophobic

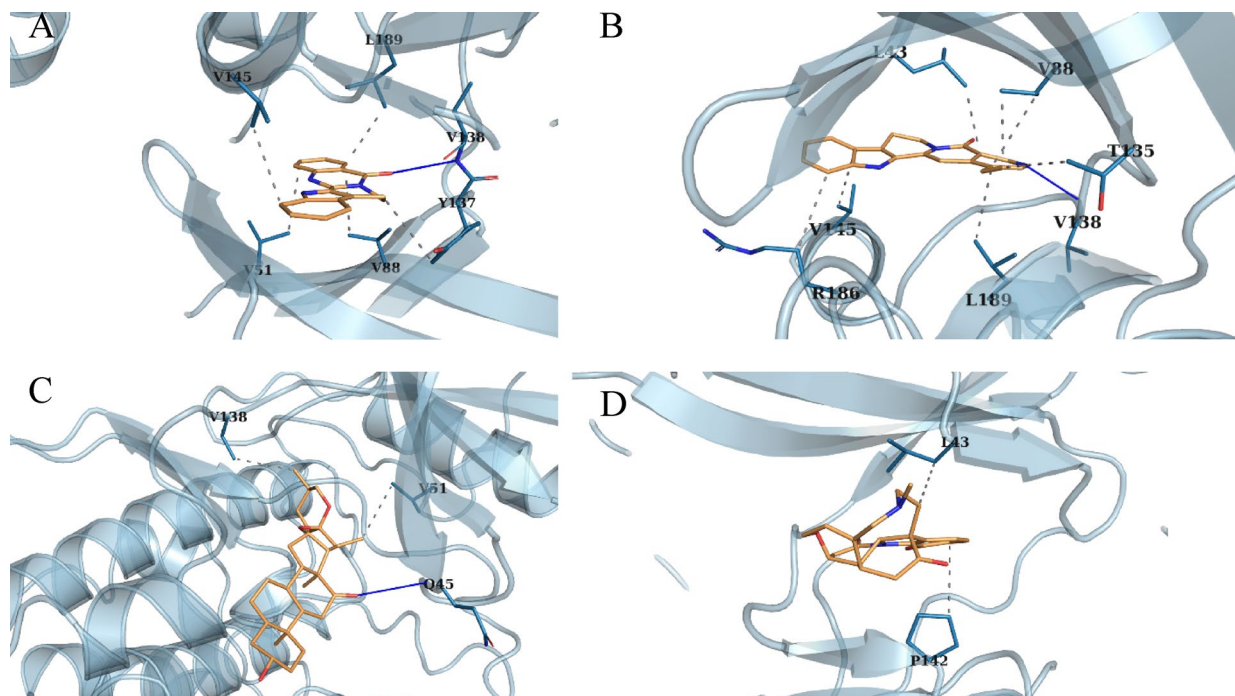


Fig. 6. TYK2 complex with (A) Rutaecarpine, (B) Angustine, (C) Hecogenin, and (D) Vomicine. The highlighted residues are marked with blue sticks. Blue lines indicate hydrogen bonds, gray dots represent hydrophobic interactions.

interactions and one H-bond (Fig. 6C)(Fig. 2C S1). ΔG_{bind} of Vomicine in complex with TYK2 was -66.73 ± 3.18 kJ/mol (Table 8) and had two hydrophobic interactions (Fig. 6D)(Fig. 2D S1). Compared with polar contributions, the results indicated that hydrophobic or non-polar contributions have the most influence on TYK2 complexes' interactions and binding energies. Our results showed that all 4 compounds can be potential inhibitors for TYK2.

In contrast to the two previously mentioned, Hecogenin indicated better results for inhibiting IL-6. This complex had two hydrophobic interactions (Fig. 7C)(Fig. 3C S1) and the ΔG_{bind} was -118.74 ± 5.61 kJ/mol (Table 9). All four compounds had good results for inhibiting IL-6. ΔG_{bind} of Rutaecarpine, Angustine, and Vomicine were -117.53 ± 2.63 kJ/mol, -102.19 ± 5.58 kJ/mol, and -79.77 ± 6.19 kJ/mol, respectively (Table 9). The interactions of these three complexes were four hydrophobic interactions and one H-bond for Rutaecarpine-IL-6 complex (Fig. 7A)(Fig. 3A S1), five hydrophobic interactions and one H-bond for Angustine-IL-6 complex (Fig. 7B)(Fig. 3B S1), and one hydrophobic interaction and two H-bonds for Vomicine-IL-6 complex (Fig. 7D)(Fig. 3D S1). Similar to TYK2 complexes, our results demonstrated that all 4 compounds had a good inhibitory potential of IL-6. The important amino acid residues of all ligand-protein complexes at the binding site are shown in Table 1S1.

Discussion

Several inflammatory molecules and mechanisms are involved in the pathogenesis of RA. However, targeting these pathways alone has not been effective for treatment without complications. Previous investigations have looked into the inhibition of each of these drug targets individually. Therefore, the simultaneous targeting of several drug targets (multi-targets) in the pathogenesis of diseases can be a novel therapeutic approach. In this study, we aimed to target different levels of inflammation (CD20, TYK2, and IL-6) for higher effectiveness and to introduce suitable drug candidates with low side effects. Also, we have constructed a compound-target-pathway network to visualize how Rutaecarpine, Hecogenin, Angustine, and Vomicine modulate interconnected rheumatoid arthritis (RA) pathways(Fig. 6S1), as depicted in the provided diagram. The network is centered on the JAK-STAT signaling pathway, the primary regulator of cytokine-induced inflammation, with IL6 and its receptor IL6ST (gp130) as hub nodes. These compounds target IL6 and its downstream effectors, including TYK2 and other interleukins (e.g., IL2, IL12A, IL17D), while the presence of CD20 (MS4A1), a B-cell surface marker with a role in immune regulation, suggests the addition to the network of the potential for B-cell modulation in RA. The connections to RA and autoimmune disease nodes suggest that these compounds, along with CD20, may influence disease progression through targeting this pathway. Since all four compounds are inhibitors of the same pathway, their simultaneous activity will have synergistic effects. By inhibiting various nodes like upstream cytokines (e.g., IL6), signaling kinases (e.g., TYK2), and downstream effectors (e.g., IL12A, IL17D), these compounds in combination will be able to more successfully suppress the overactivation of the JAK-STAT pathway than any single inhibitor. For instance, Rutaecarpine's blockage of IL6 can prevent initial pathway activation, while Hecogenin's action on TYK2 and Angustine's and Vomicine's downstream activities

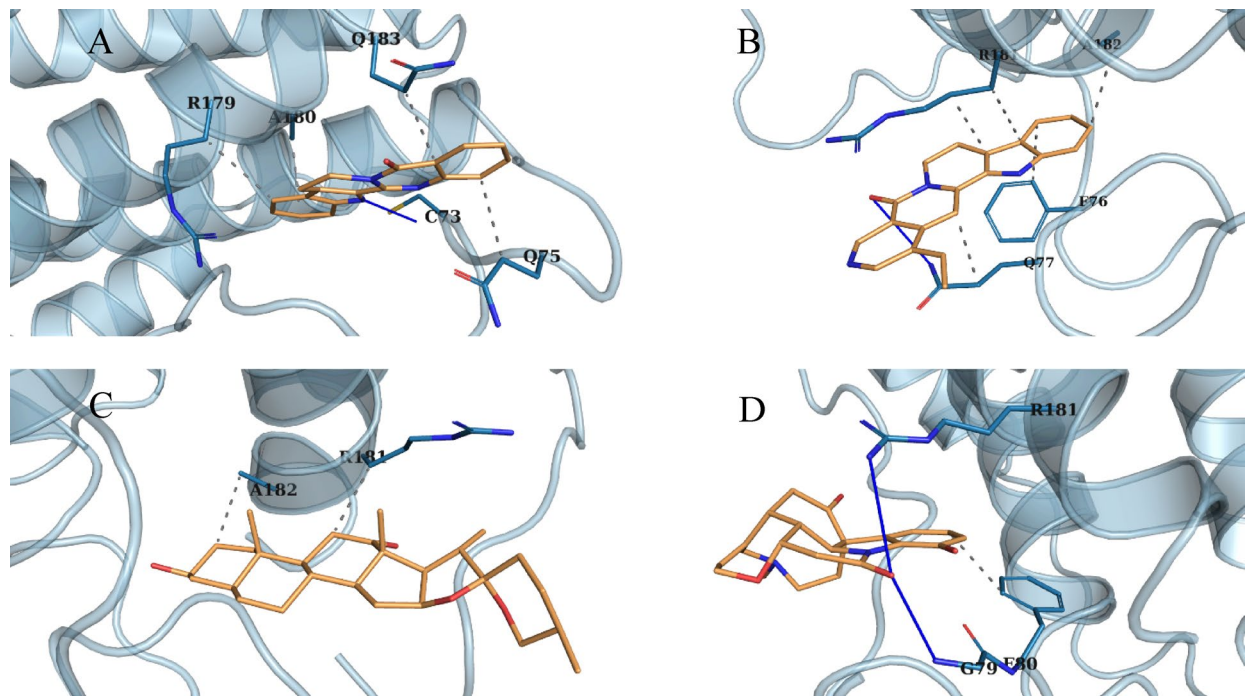


Fig. 7. IL-6 complex with (A) Ruteacarpine, (B) Angustine, (C) Hecogenin, and (D) Vomicine. The highlighted residues are marked with blue sticks. Blue lines indicate hydrogen bonds, gray dots represent hydrophobic interactions.

further ensure that the inflammatory cascade is blocked at multiple points. This collective inhibition can enhance therapeutic activity, reduce the amount of individual compounds needed, and minimize the risk of resistance or compensatory pathway activation.

IL-6 is one of the most important innate immune cytokines that is secreted from activated macrophages in RA, and its level is directly related to the severity of joint destruction²². In 2022, a study was conducted to identify new IL-6 inhibitors. The best docking score was -27.57 kJ/mol (-6.5 kcal/mol) for the compound ZINC04256801. The residues involved in this complex were Phe78, Leu178, Arg179, and Arg182⁵⁹. In the study conducted by Tran et al., the combination of DB12903 with a docking score -25.02 kJ/mol (-5.9 kcal/mol) and DB08402 with a docking score -20.74 kJ/mol (-4.9 kcal/mol) was introduced as their best drug candidate. The residues involved in this complex were Phe74, Gln75, Arg179, and Gln183⁶⁰. But our candidate compounds showed better results as mentioned above, docking score for Ruteacarpine, Angustine, Hecogenin, and Vomicine was -7.0 , -7.3 , -7.1 , and -7.4 kcal/mol. But there is similar residues in the study conducted by Tran et al. and our study. Residues involved in our complexes were F74, Q75, R179, and Q183.

Tyrosine kinase 2 (TYK2) is a member of the Janus kinase (JAK) family, which plays a role in the signaling pathway of interleukin (IL) 12, IL-23, and type I interferons (IFN-I)¹⁴. In our study, we focused on the binding of the four candidate compounds to the pseudokinase (JH2) domain of TYK2, which is known to be an allosteric site distinct from the canonical ATP-binding kinase domain (JH1). Our molecular docking and dynamics simulations confirm that Deucravacitinib and all four candidates preferentially bind to the JH2 domain, consistent with Deucravacitinib's known allosteric mechanism of action⁴⁵. Due to the importance of TYK2 in inflammatory cytokine signaling, it became a promising target in inflammatory diseases. A study conducted in 2016 to find new inhibitors for TYK2 among 2924 drug compounds in the ZINC drug database found three compounds, including ZINC12503271 (Gemifloxacin), ZINC05844792 (Nebivolol), and ZINC00537805 (Glyburide), that were introduced as specific inhibitors of TYK2. They accomplished 25 ns MD simulations, and all RMSD values were between 0.075 nm and 0.275 nm. We had 150 ns MD simulations, and the RMSD values of TYK2 in all complexes were between 0.38 nm and 0.55 nm.

CD20 is one of the B lymphocyte surface markers that plays a role in the development and differentiation of B lymphocytes into plasma cells²⁸. ACPAs produced by plasma cells have important roles in the pathogenesis of RA^{24,25,61}. It is expected that the development and differentiation of B-cells into plasma cells will be prevented when the candidate compounds bind to the binding site of the Rituximab-CD20 complex, which requires more experimental studies in this field²⁸. So far, no study has been conducted to identify small molecules that inhibit CD20, but several studies have been conducted to evaluate the anti-CD20 monoclonal antibody treatment. A single course of two infusions of rituximab, alone or in combination with either cyclophosphamide or continued methotrexate, provided significant improvement in RA symptoms at both weeks 24 and 48⁶². Hauser et al. investigate the Efficacy and safety of four years of Ofatumumab treatment in relapsing multiple sclerosis. Their results show that the Ofatumumab treatment group had fewer new/enlarging T2 lesions (annualized rate 0.08), low numbers of gadolinium-enhancing (Gd+) T1 lesions (0.01 lesions/scan), and had a low annualized

relapse rate (ARR 0.05 (95% confidence interval: 0.04–0.07))⁶³. Our results introduced 4 compounds, including Rutaecarpine, Hecogenin, Angustine, and Vomicine, as new drug candidates for the treatment of RA. Rutaecarpine, Hecogenin, and Angustine inhibit all three targets with high affinity and stability, while Vomicine inhibits both TYK2 and IL-6 and can't inhibit CD20.

Rutaecarpine is a pentacyclic indolopyridoquinazolinone first isolated from *Evodia rutaecarpa*, one of the most famous traditional Chinese medicinal plants for the treatment of various diseases^{64,65}. Many articles have mentioned the therapeutic effects of Rutaecarpine. In a study conducted by Nie et al. in 2016, it was found that this alkaloid can be effective in improving hyperlipidemia and hyperglycemia in rats by regulating the IRS-1/PI3K/Akt and AMPK/ACC2 signaling pathways⁶⁶. In addition, Wan et al. showed that Rutaecarpine improves the healing of osteoarthritis by inhibiting PI3K/AKT/NF- κ B and MAPK signaling pathways in mice⁶⁷. Er Miao San (EMS) is a herbal formula of traditional Chinese medicine that can play a role in improving RA by containing Quercetin, wogonin, and Rutaecarpine. Furthermore, it can improve RA by targeting TNF- α , IL-6, and IL-1 β ⁶⁸. It was also shown that EMS has an inhibitory effect on the production of NO, TNF- α , and IL-6 from RAW264.7 cells stimulated with lipopolysaccharide (LPS)⁶⁸. Inhibition of different signaling pathways in inflammation by Rutaecarpine, and also, the long history of using it in traditional medicines, shows that this compound, in addition to being safe, can be effective for the treatment of inflammatory diseases such as RA in the future. In this study, Rutaecarpine had promising results. As mentioned above, Rutaecarpine inhibited CD20, TYK2, and IL-6 by docking score of -7.3 , -10.2 , and -7.0 and by ΔG_{bind} of -44.40 ± 1.89 , -96.59 ± 4.78 , and -117.53 ± 2.63 , respectively.

Another compound with a high affinity for all three drug targets was Hecogenin, which is a steroidal saponin. Many studies have investigated its therapeutic effects. This compound is found in large amounts in *Agave* species⁶⁹. A study conducted by Gilberto Santos Cerqueira and colleagues on models of gastric ulcers in mice showed that the administration of Hecogenin can have strong protective effects on the stomach, which seems to be mediated by the opening of K⁺_{ATP} channels and the COX-2/PG pathway⁷⁰. Additionally, the effects of hecogenin in the rat model of RA have also been investigated⁷¹. Treating mice with hecogenin significantly reduced paw edema, arthritis score, and joint diameter. In the histopathological and radiological analyses of the ankle joint, it was also found that Hecogenin treatment prevents joint destruction⁷¹. Similar to Rutaecarpine, Hecogenin inhibited all three targets with good affinity. It inhibited CD20, TYK2, and IL-6 by docking scores of -7.2 , -7.2 , and -7.3 and by ΔG_{bind} of -35.06 ± 4.36 , -95.27 ± 3.72 , and -118.74 ± 5.61 , respectively. Studies mentioned above and our results indicated that Hecogenin is one of the most important small molecules with the potential of being a candidate compound for future RA drugs.

Vomicine is one of the main components of the medicinal composition of *Semen strychni* and is used in traditional Chinese medicine. In an in vivo study conducted by Yingshi Zhan and his colleagues, it was found that Vomicine has neuroprotective and neuroinflammatory effects in the treatment of ALS⁷². Based on the results obtained, Vomicine may reduce neuroinflammation by regulating the cGAS-STING-TBK1 pathway, making it a potentially effective treatment option for ALS⁷². In contrast to Rutaecarpine, Hecogenin, and Angustine, Vomicine couldn't inhibit CD20, but it was good at inhibiting TYK2 and IL-6 by docking scores of -8.7 and -7.4 , respectively. However, Angustine was our best compound for inhibiting CD20 and TYK2 by ΔG_{bind} of -60.40 ± 3.54 and -117.36 ± 5.73 . Also, it was good at inhibiting IL-6 by ΔG_{bind} of -102.19 ± 5.58 .

Conclusion

In recent years, computer-aided drug design projects have been carried out to develop novel inhibitors for various diseases in a short time and cost-effectively. RA, a disease with a complex inflammatory pathology, has also received a lot of attention from researchers in recent years to design and produce new inhibitors. Although many inhibitors have been identified for the therapeutic purposes of RA, no study has been conducted to identify potential compounds for the simultaneous targeting of various proteins, such as IL6, TYK2, and CD20, which are at different levels of RA pathogenesis. In this study, we used a multi-target strategy and selected Rutaecarpine, Hecogenin, Angustine, and Vomicine compounds for simultaneous inhibition of the IL6, TYK2, and CD20 targets. Rutaecarpine, Hecogenin, and Angustine inhibited all three targets with high affinity and stability, while Vomicine inhibited both TYK2 and IL-6 and couldn't inhibit CD20. We hope that the results of this research, with future experimental studies of candidate compounds, will provide a new treatment approach for RA.

Data availability

All the data generated in this article are available from the first author on request.

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Declarations

Competing interests

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