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Regular paper

Investigation of VEGF (rs 699947) polymorphism in the progression of Rheumatoid Arthritis (RA) and *in-silico* nanoparticle drug delivery of potential phytochemicals to cure RA

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Mutation in the *VEGF* gene disturbs the production of chondrocytes and angiogenesis which are essential for cartilage health. Cytokines and chemokines produced by auto-activation of B-cells degrade cartilage. Bruton's Tyrosine Kinase (BTK) plays a crucial role in the activation of these B-cells. VEGF has a central part in angiogenesis, in the recruitment of endothelial cells, and is involved in mechanisms that result in tumour formation. The objective of this research is to investigate the potential role of *VEGF* polymorphism in the development of Rheumatoid Arthritis (RA) and the screening of potential natural, synthetic BTK inhibitor compounds as possible *in-silico* chemotherapeutic agents to control auto-activation of B-cells and cartilage degrading cytokines. In this study, it had been shown that allele A frequency was significantly higher than that of allele C in RA-positive patients as compared to controls. Hence it depicts that allele A of *VEGF* (rs699947) can increase the risk of RA while allele C has a protective role. The phytochemicals which showed maximum binding affinity at the inhibitory site of BTK include beta boswellic acid, tanshinone, and baicalin. These phytochemicals as BTK inhibitor give insights to use them as anti-arthritic compounds by nanoparticle drug delivery mechanism.

Keywords: angiogenesis, phytochemicals, Bruton's tyrosine kinase, Rheumatoid Arthritis, *VEGF* gene

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Abbreviations: BTK, Bruton's Tyrosine Kinase; RA, Rheumatoid Arthritis

INTRODUCTION

Rheumatoid Arthritis is an inflammatory auto-immune disorder of joints (van Delft & Huizinga, 2020). The inflammatory process enhances as new blood vessels originate and supply different growth factors, oxygen,and cytokines to the cells which leads to the development of irregular fibrous tissues and ultimately causesthe destruction of joints (Nakkala *et al*., 2021). A ratio of 1:3 between male and female susceptibility can be seen in previous studies. The worldwide prevalence of RA is 0.5–1% depending on geographical changes. The studies conducted in Pakistan and India represent the RA prevalence as 0.5% and 0.2–1% respectively (Bahari *et al*., 2021). Population diversity is responsible for the higher prevalence of RA in India (Otón & Carmona, 2019). There are some influential factors of RA that play a significant role in the susceptibility of RA like genetic factors and environmental factors. Almost 60% of the susceptibility is caused due to genetic factors. Although further studies are required to explain the role of environmental factors in the onset of RA, however, it is reported that smoking, dietary contents, micro-organisms, and silica exposure have a detrimental effect and they can also exacerbate the condition of RA patients (Ganesan *et al*., 2020). Dietary contents may also have a role in the severity of the disease. The majority of patients claimed that eating foods like dairy, red meat, green leafy vegetables, etc. made their medical state worse. Red meat contains high amounts of protein and fats whichenhance the pro-inflammatory process resulting in increasedseverity of the disease in patients with RA (Karami *et al*., 2019).

There are evidence and studies which support that many autoimmune ailments like rheumatoid arthritis along with systemic lupus erythematosus (SLE) and multiple sclerosis are linked with the *VEGF* gene (Martins & Fonseca, 2019). *VEGF* gene has eight exons with alternate splicing, and it is present on chromosome 6p 12. During embryonic developmental stages, the multiplication and migration of endothelial cells are induced by VEGF, and have crucial involvement in tumour development, angiogenesis, and wound healing (Jutley *et al*., 2017). In the synovial fluid of obstructed joints of RA patient, enhanced expression of VEGF is observed. The enhanced expression also results in inflammation, changes in joints, and different pathological conditions in patients along with angiogenesis. The severity of the disorder or disease can be determined by the VEGF level in the serum of patients. An elevated level of VEGF has been recorded in patients suffering from RA as compared to controls (healthy individuals) and osteoarthritis patients (Philippou *et al*., 2021). SNPs that are known and also illustrate a significant role in the expression of VEGF proteins are VEGF-2578 A/C (rs 699947), +405 G/C, –460 C/T +936 C/T (Aterido *et al*., 2017). According to a study in Korea, an association is seen between VEGF SNPs and RA (Qindeel *et al*., 2020).

Although VEGF is very significant to proliferate cartilage and bone cells but in RA auto-activation of B-cells produces chemicals that damage these cells so, this auto-activity of the B-cells must be controlled (Goswami *et al*., 2022). Their activation is mediated by a regulatory protein Burton's Tyrosine Kinase. This protein stimulates B-cells to produce cytokines and chemokines. In a healthy individual, synoviocytes produce lubrication and nutrition for the surrounding cartilage tissue, and the synovium has a minimal number of cellular infiltrates. In RA, B-cells infiltrate the synovium and release cytokines, chemokines, and enzymes that promote joint deterioration and inflammation (Wang *et al*., 2021). It causes the synovium to enlarge, causing synoviocyte production and inflammatory cell penetration to form a pannus that eventually invades the nearby bone and cartilage. B-cell receptor-dependent cell proliferation is inhibited by BTK inhibition, which ultimately results in a decrease in inflammatory cytokines. Targeting the BTK's activity may therefore result in the loss of B cell signaling, which in turn may open up a treatment option for RA. The cytoplasmic, non-receptor tyrosine kinase BTK transmits signals via numerous cell surface molecules. All hematopoietic cell types, except for T, NK, and plasma cells, express it (Miao *et al*., 2020). BTK communicates with chemokine receptors, Fc receptors, Toll-like receptors, B cells receptors, CD40, and B cells receptors. These activated proteins damage tissues, especially joints. A high level of BTK causes autoimmunity while a low level of BTK helps in improving autoimmune diseases like RA (De Vries *et al*., 2019). The abnormal activity of BTK might be ceased via various inhibitor synthetic as well as natural bioactive compounds. The core aim of this study was to find out VEGF SNP in the Pakistani population and possible chemotherapeutic drugs.

METHODOLOGY

Sampling

Samples of blood from 100 healthy individuals (controls) and 100 RA patients who were positive for Rheumatoid Factor were collected from two major cities of Pakistan. These blood samples were collected from D.H.Q Hospital Toba Tek Singh, Sheikh Zaid Hospital, Lahore, and Fatima Memorial Hospital Lahore. The participant's approval was taken on an informed consent form and their disease history was recorded on a Performa (covered all aspects related to disease and the risk factors associated with RA). Afterwards the experimental work was carried out at the Institute of Microbiology and Molecular Genetics of the University of the Punjab, Lahore-Pakistan.

IRB approval

The ethical approval for this research study was granted by Departmental Research Ethics and Biosafety Committee under the issuance certificate D/2302/MMG, dated 6-12-2019.

DNA Isolation and Amplification

The fast DNA extraction method makes it simpler to extract DNA from blood and produces a significant volume of non-degraded DNA. The application of triton X-100 prevents DNA degradation (Shahraki *et al*., 2022). $MgCl_2$ acts as a buffering agent in the red blood cell lysis buffer, whereas EDTA acts as a chelating agent, stops DNA deterioration, and concentrates DNA. Nuclear lysis buffer and red cell lysis buffer both lysed red blood cells, break the nuclear wall and release the nuclear material inside. SDS was added to the lysis buffer to solubilize proteins and lipids, and chloroform was used to breakdown proteins and purify DNA. Chloroform was used to separate the organic phase from the aqueous phase while maintaining DNA protection in the latter. The precipitation of DNA was accomplished using ethanol. The first procedure involved transferring 500 µl of blood into an Eppendorf tube and then adding 1000 µl of RBC lysis buffer. The Eppendorf was centrifuged for 2 minutes at 7000 rpm(revolutions per minute) after being lightly inverted or gently shaken. After that, the supernatant was removed, and the Eppendorf tube containing the pellet received another 1000 µl addition of RBCs lysis buffer. Once more, the particle was centrifuged at 7000 rpm for 2 minutes, three to four times, until the haemoglobin was eliminated. After RBC lysis buffer was added, the tube was vortexed to break up the pellet and remove the haemoglobin, revealing that the particle exclusively contained WBCs. The tube was then positioned downward on the tissue paper for a brief period of time. The Eppendorf tube was then filled with 600 µl of chloroform (kept at 4°C), 400 µl of nucleic lysis buffer, 100 µl of NaCl, and 400 µl of lysis buffer (kept at 4°C). After that, centrifugation took place for two minutes at 7000 rpm. The result was two phases, an organic phase and an aqueous phase, separated by a layer. Then, 800 µl of 100% ethanol (pre-chilled and maintained at –20°C) was added to the Eppendorf tube containing the supernatant after 400 µl of the supernatant had been emptied into another Eppendorf tube. After the vortex, the DNA manifested itself in the aqueous phase as a white thread-like structure. The tube was centrifuged at 12000 rpm for one minute, the supernatant was removed, and the tube was dried at room temperature (on tissue paper). After adding 100 µl of T.E. buffer, the tube was vortexed. The DNA-filled Eppendorf tube was then kept at –20°C. The stock solution for the primers was created using a 1:10 ratio.

- Forward inner primer (A allele) "GCCAGCTGTAGGCCAGACCCTGGT"
- Reverse inner primer (C allele) "TCAGTCTGATTATCCACCCAGACCG"
- Forward outer primer "GTGCACGAATGATGGAAAGGGAGG"
- Reverse outer primer "CCCCATCCCATTCTTGCATATAGG"

50 µl were used to conduct the PCR reaction. Forward and reverse primers, genomic DNA, 10 µl of nuclease-free water, and 25 µl of the master mix were all added. The DNA strand was first denaturized at 95°C for 5 minutes, and then it was finally denaturized at 95°C for 30 seconds. For rs699947, the DNA strand was annealed at 67.3°C for 30 seconds, extended at 72°C for 1 minute, and finally extended for 5 minutes at 72°C. The PCR was run for 30 cycles. The PCR products had lengths of 183 bp and 299 bp for rs 699947 and were kept at a temperature of 4°C. On electrophoresis on 1.3% agarose gel, the amplified products were seen. The size of the PCR products was measured using Thermo Scientific's quick ruler low-range DNA ladder (SM1103).

In-Silico Screening of BTK Inhibitor Chemicals

Plants have a variety of bioactive compounds that can block the activity of BTK to control the auto-activation

Table 1. The laboratory parameters and disease activity in association with VEGF

Parameters	AA		CA + CC		*P*
	N	Median (IQR)	N	Median (IQR)	
Age (Years)	71	47 (20–60)	29	45 (20–60)	0.014*
Duration of Disease (Years)	63	7 (6 months – 15 Years)	24	7 (6 months – 15 Years)	0
CRP (mg/L)	47	12 (6–30)	25	14 (6–34)	0.049*
Number of Tender Joints	56	5 (2–9)	22	7 (2–11)	0.105
Number of Swollen Joints	51	3 (0–5)	18	2 (0 –8)	0.126
ESR (mm/h)	23	34 (18–50)	28	23 (16–48)	0.121
	AA		CA + CC		*P*
	N	n (%)	N	n (%)	
Anti – Cyclic Citrullinated Peptide Antibody (CCP)	54	30 (55%)	25	14 (56%)	0.222
RF Presence	71	71 (100%)	29	27 (93%)	0.269

*P<0.05, Significant; P>0.05, non-Significant

of the B-cell, chemokines, and cytokines. Screening of these phytochemicals has been done by using computational tools and online databases. To avoid a huge number of resources, funding, and wastage of time to sort out a particular chemical as a BTK inhibitor, in-silico screening is the best approach to screen out target bioactive chemicals. The target protein BTK was found in PDB to have a 3D structure (ID-6J6M). Using PyMol (version 2.5.2), water molecules and already-bound ligands were removed (Hari, 2017). Target ligand information was gathered from a variety of sources, and 3D ligand structures were acquired in SDF format from online chemical databases like Zinc 15, PubChem, and CHEMBL database. Then, using Swiss ADME and Lipinski's Rule of Five, the ligands' drug resemblance was evaluated. Online SMILES Translator (nih.gov) was used to convert the SDF format of the ligands to the PDB format, and Auto Dock Vina (version 1.5.7) was used to convert the PDB to the PDBQT format and then docking by using the command prompt (Samdani & Vetrivel, 2018).

RESULTS

Patients were diagnosed according to the criteria defined AmericanCollege of Rheumatology (ACR) in 2010. Among those 100 patients, males were only 33% while females constitute a major proportion (67%) of the study. The common symptoms in RA patients, whose blood samples were used, found morning stiffness (82%), tenderness in the joints (78%), fatigue (25%), severe pain in joints (85%), fever (20%), and joint Swelling (63%) (Fig. 1) (Ganesan *et al.*, 2020). The risk factors associated with RA were genetic disorder (9%), smoking (7%), diet (33%), hormonal changes (17%), other infectious diseases (6%), and no response (28%) (Fig. 2).

Single Nucleotide Polymorphism in DNA due to extrinsic or intrinsic factors has both deleterious and beneficial effects depending on its nature. In this study, a base change in the VEGF gene in different populations showed significant results in the progression of rheumatoid arthritis. In Table 1, a possible linkage between VEGF polymorphism with RA disease activity was analyzed based on the demographic and clinical condition of the patients. The alleles of the VEGF gene were correlated with disease duration, rheumatoid factor, age of patients and C-reactive protein level.

The appearance of 183bp DNA bands on agarose gel electrophoresis indicated A allele (Fig. 3) and 299bp DNA bands represented C allele (Fig. 4), and the DNA ladder used was Fermantas SM1103. In VEGF, C allele functions as a major allele and A allele as a minor allele. The frequencies of genotype and allele were considerably different in patients and controls. The percentage of AA genotype in RA patients and in controls was 71% and 39% respectively. However, the percentage of CC

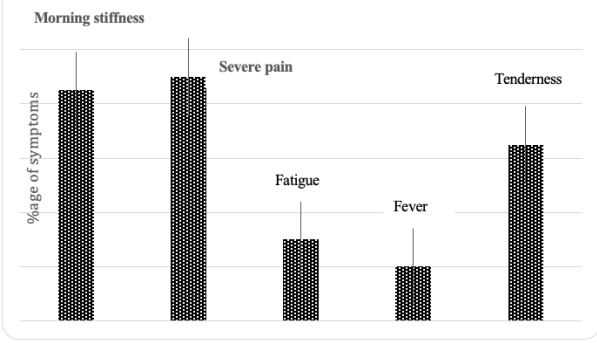

Figure 1. Symptoms reported by Rheumatoid Arthritis

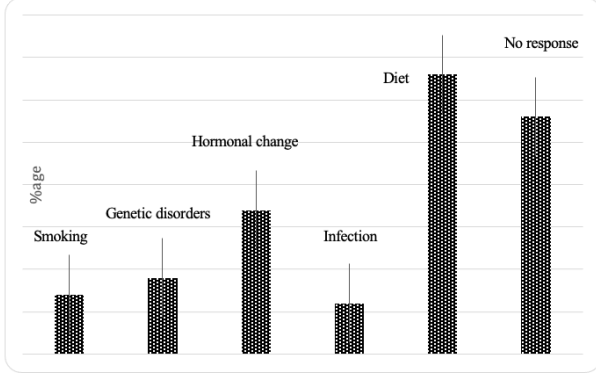

Figure 2. Risk factors involved in progression in RA patients

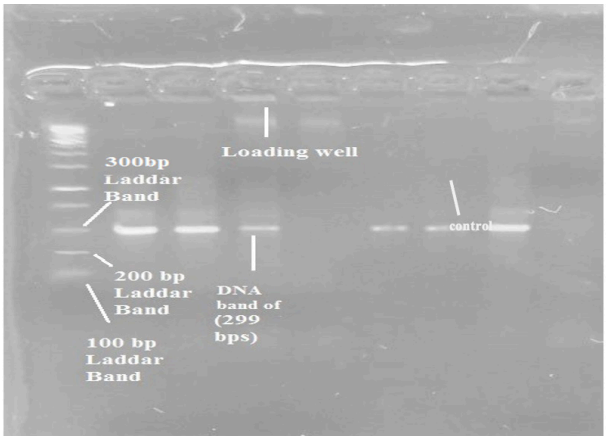

Figure 3. Amplified DNA band of our interest (183bp) shown under the UV Illuminator

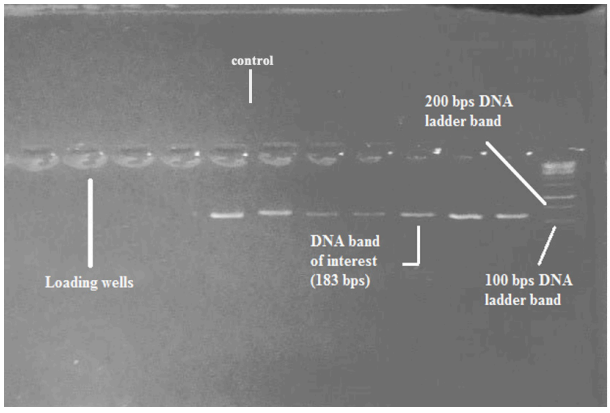

Figure 4. Amplified DNA band of our interest (299bp) shown under the UV Illuminator

genotype in RA patients and control was 23% and 52% respectively.

Moreover, in heterogeneous conditions where both alleles (A and C) were present, the percentage of AC genetic constitution in patients and controls was 06% and 09% respectively. Hence, the above data endorsed that VEGF A/C polymorphism hasa role in association with the risk of rheumatoid arthritis. Table 2 and Table 3 revealed different frequencies of genotypes and frequencies of alleles in controls and patients. Our findings suggested that the AA genotype of VEGF was observed considerably higher in RA patients but lower in healthy individuals that depicted the role of SNPs in the severity of RA.

The chi-square test was done by keeping a degree of freedom (df) value 2, the *p*-value was 0.034 which is less than 0.05, and the regression value was 0.461. All these values showed that the VEGF gene SNP has a significant influence on rheumatoid arthritis progression.

Bioactive Compounds to Inhibit BTK Activity

Plants have a variety of bioactive compounds capable of curing rheumatoid arthritis. These compounds can be screened out in-silico by using different computational tools. The results of docked compounds have been given in Tables 4 and 5 showing binding affinities to block BTK. Protein-ligands docking was done by targeting THR 474, GLU 475, MET 477, and CYS 481 residues of BTK. The results were observed in PyMol (Version: 2.5.2) and noted binding affinities in KJ/mol shown in Fig. 5. The Pleckstrin homology (PH) domain, the Tec homology (TH) domain, the Src homology (SH3) domain, the SH2 domain, and the C-terminal region containing kinase activity are the five sections that make up BTK, according to its structures. BTK inhibitors can be divided into two categories based on how they bind to BTK and how their chemical scaffold structures and mechanisms of action. The amino acid residue CYS 481 in the ATP-binding region of BTK is a covalently bonded target for irreversible BTK inhibitors (Wang *et al.*, 2021). The other class of BTK inhibitors are reversible inhibitors, which bind to an inactive form of the kinase by accessing the particular SH3 pocket of BTK. In order to treat RA, phytochemicals are delivered as drugs via nanoparticles, acting as a BTK inhibitor (Fig. 6).

DISCUSSION

The VEGF gene has a prominent role in the onset of many other autoimmune diseases. There are almost thirteen studies that depict the evidence that *VEGF* was higher in RA patients in contrast to healthy individuals, and hence are in favour of an association between RA and the *VEGF* gene. But contrary to that there are also many studies that deny this and report no association among *VEGF* and the risk of RA like + 936 T/C, *VEGF*-634 C/G, -1154 A/G and –2578 A/C polymorphisms have no relation with RA. In another study, a significant association was observed between *VEGF* (rs 699947) SNPs and RA (Paradowska-Gorycka *et al.*, 2016). The studies show a relationship between the *VEGF* gene and RA in terms of severity along with overlap-

Table 2. Genotypes and Allele frequencies of VEGF C/A polymorphism

Gene mutation	Genotypes	Genotype frequency cases (n=100)	Genotype frequency controls (n=100)	Alleles	Allele frequency cases (n=100)	Allele frequency controls (n=100)
VEGF *rs*699947	CC	23	52	C	52	113
	AA	71	39	A	148	87
	CA	06	09			

Table 3. Analysis of the VEGF gene SNP rs699947 by Chi-square test

Subjects	Homozygous CC	Heterozygous CA	Homozygous AA	Total	X^2 df = 2	*p*-value	Regression
Cases	23	06	71	100	23.32	0.034	0.461[a]
Controls	52	09	39	100			

Table 4. Potential Phytochemicals Inhibitors of Burton's Kinase (BTK)

Sr. No	Compound name	Binding affinity (KJ/mol)	Configuration
	BETA-BOSWELLIC-ACID	–8.4	ASN 479 (CN-OH,3.1)
	TANSHINONE	–8.3	MET 477 (CO-OH,3.0) ALA 478 (CO-OH,3.4)
	BAICALIN	–8.2	CYS 481 (CN-OH,2.9) ASN 479 (CN-OH,2.3) ASN 484 (CN-OH,3.1)
	BERBERASTINE	–8.1	MET 477 (CN-OH,3.2)
	APIGENIN	–7.8	MET 477 (CN-OH,3.3) ASN 484 (CN-OH,2.9) THR 410 (CN-OH,3.1)
	6-DEOXYJACAREUBIN	–7.8	LYS 430 (CN-OH,3.3) MET 477 (CN-OH,3.2) MET 477 (CO-OH,2.4)
	(-)-ALPHA-BISABOLOL	-6.3	LYS 430 (CN-OH,3.0)
	QUERCETIN	–7.6	MET 477 (CN-OH,3.0) MET 477 (CN-OH,3.2) MET 477 (CN-OH,2.4) THR 474 (CO-OH,2.9) THR 410 (CN-OH,3.1)
	SPEBRUTINIB	–7.5	ASN 484 (CN-CO,3.0) MET 477 (CO-NH,2.5)
	CEPHARANOLINE	–7.4	CYS 481 (CN-OH,3.1) THR 410 (CO-OH,2.1)

Table 5. Synthetic BTK Inhibitors

Sr. No.	Phytochemical	Binding Energy (kJ/mol)	Configurations (PyMol)
1.	RN-486	–7.9	CYS 481 (CN-CO,3.0) LYS 430 (CN-OH,3.1) ASN 479 (CO-OH,3.2)
2.	BARICITINIB	–7.6	MET 477 (CO-NH,3.5) MET 477 (CO-NH,2.5) THR 410 (CO-SO,3.1)
3.	SPEBRUTINIB	–7.5	ASN 484 (CN-CO,3.0) MET 477 (CO-NH,2.5)
4.	FENEBRUTINIB	–7.0	MET 477 (CN-OH,3.1) MET 477 (CO-OH,2.7)
5.	IBRUTINIB	-7.3	MET 477 (CO-NH,3.2) LEU 408 (CO-NH,3.5)
6.	SPEBRUTINIB	–7.5	ASN 484 (CN-CO,3.0) MET 477 (CO-NH,2.5)
7.	TIRABRUTINIB	–6.9	ASN 484 (CN-OH,3.4)
8.	UPADACITINIB	–5.8	ASN 484 (CN-CO,3.3)
9.	ZANUBRUTINIB	–6.5	CYS 481 (CN-CO,3.5) LYS 430 (CN-OH,3.1)
10.	ACALABRUTINIB	–5.0	GLU 488 (CO-CO,2.8) ALA 478 (CO-NH,3.5)

ping of various other disease parameters i.e., activity of disease, functional disability, and joint damage. The main objective behind this work was to access the risk of single nucleotide polymorphism of the *VEGF* gene in RA patients. Two alleles A and C were studied, and their frequency was determined. It had been shown that allele A frequency was significantly higher than that of allele C in RA-positive patients. On the other hand, controls have more frequency of allele C as compared to allele A. Hence it depicts that allele C plays a minor role in the risk or severity of the RA disease. Recent studies also indicated that allele A of *VEGF* (rs699947) can increase the risk of RA; however, only a protective role is seen in the case of allele C (Saravani *et al.*, 2019). A low level of VEGF is due to allele A and its genotype AA can be its risk factor. While in the case of atherosclerosis, allele C has a protective effect. A relationship has been observed between low serum level and the AA genotype of VEGF in RA patients, as illustrated by a cohort study of 419 patients (Laurindo *et al.*, 2022). Similarly, another

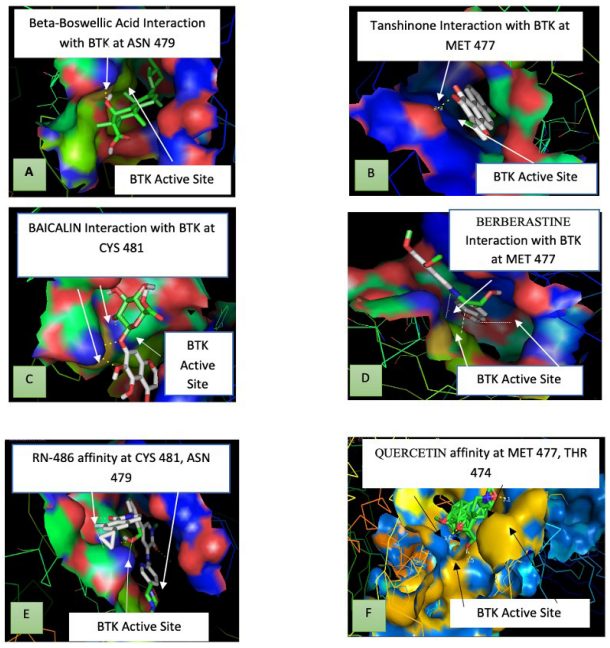

Figure 5. BTK inhibition interactions with different chemicals; (A) BETA-BOSWELLIC ACID, (B) TANSHINONE, (C) BAICALIN, (D) BERBERASTINE, (E) RN-486, (F) QUERCETIN

research study elucidates that functional polymorphism of VEGF (A/C) may increase the risk of RA in older and Anti-Citrullinated Peptide Antibody negative patients (Arleevskaya *et al.*, 2021). There are some other factors that may be involved in the progression of RA like auto-activation of BTK protein, age, and CRP (Alam *et al.*, 2017).

Rheumatoid arthritis is a multifactorial joint disorder, and VEGF SNP is one of those factors at a genetic level and abnormally activated BTK protein is another one. Only we found that mutation in this gene causes disturbance in angiogenesis proliferation and cartilage repair which is the major possible effect of a mutated VEGF gene. On the other hand, targeting the BTK protein is a phenotypic therapeutic approach which is involved in the destruction of joint lining. So, it is better to cease the damaging effects of this abnormally activated BTK protein to protect the remaining joints' assets. The effect of SNP on the VEGF product protein is the formation of abnormal heparin-binding protein which does not involve in repairing the cartilage and in the proliferation of new blood vessels (Huang and Wang, 2020).

Tanshinone showed binding affinity -8.3 (kJ/mol) at MET 477 (CO-OH,3.0), and ALA 478 (CO-OH,3.4). This chemical is extracted from red sage or commonly known as behmansurkh which is a root of *Salvia miltiorrhiza* and belongs to the Labiatae family of plant kingdom native to China and Japan (Li *et al.*, 2021). For 2000 years ago in China, it is also being used to treat cardiovascular and cerebrovascular disorders, functions of blood flowing into the heart and liver by removing blood stasis according to Traditional Chinese Medicine (TCM) (Deng *et al.*, 2019).

Baicalin showed binding energy of -8.2 (kJ/mol) at CYS 481 (CN-OH,2.9), ASN 479 (CN-OH,2.3), ASN 484 (CN-OH,3.1) residues. It is a flavonoid phytochemical obtained from the root of *Scutellaria baicalensis* belonging to the mint family of flowering plants. This plant is commonly known as Chinese Skullcap or Huangqin. It has powerful antioxidant properties greater than ascor-

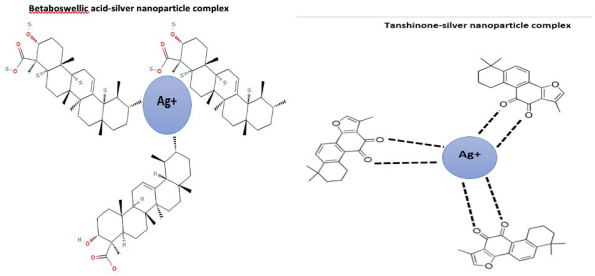

Figure 6. Phytochemicals used *via* nanoparticle drug delivery as BTK inhibitor to cure RA

bic acid which can scavenge toxic free radicals like superoxide ion, hydroxyl ion, hydroquinone, and polycyclic aromatic compounds (Shakya *et al.*, 2023). It has anti-inflammatory, anti-allergic, anti-bacterial, anti-hypertensive, and food condiment (Ma *et al.*, 2021). Berberastine showed binding energy of –8.1 (KJ/mol) at MET 477 (CN-OH,3.2). It is a phytochemical obtained from the rhizome of *Hydrastis canadensis* belonging to the Ranunculaceae family of angiosperm plants. It is utilized as a yellow dye, astringent, and insect repellent. Goldenseal contains various alkaloids like hydrastine, berberine, canadine, tetrahydroberberastine and berberastine which have great pharmaceutical importance (Brullo *et al.*, 2021).

RN-486 is a synthetic chemical under development which showed binding energy of –7.9 (kJ/mol) at CYS 481 (CN-CO,3.0), LYS 430 (CN-OH,3.1), and ASN 479 (CO-OH,3.2). It binds directly to CYS 481 amino acid to inhibit the enzyme, Bruton's Tyrosine Kinase. RN-486 inhibits inflammation of joints and other systemic inflammation alone or along with methotrexate. It potentially inhibits BTK as small molecule disease-modifying drugs to treat rheumatoid arthritis and other auto-immune disorders (Sakthivel and Habeeb, 2018). It is a potent reversible BTK inhibitor. It also blocks the signals of BCR to inhibit phosphorylation of both BTK and PLC-2 in B-cells. RN-486 shows similar activities in both human and rodent models to prevent type I and type III hypersensitivity responses effectively (Arneson *et al.*, 2021). Ibrutinib is a commercially available synthetic drug, but it has some side effects like diarrhea, upper respiratory tract infection, bleeding, fatigue and cardiac side effects. But RN-486 is a synthetic and under development chemical which has only in-silico trials as compared to phytochemicals which showed more binding affinity than RN-486 to inhibit BTK. Moreover, synthetic drugs take more time and expense, but phytochemicals are ready-made with less or no side effects at the very cheapest cost and can be extracted from plants (Aziz *et al.*, 2023a; Naveed *et al.*, 2023a; Naveed *et al.*, 2023b; Naveed *et al.*, 2023c; Naveed *et al.*, 2022a; Naveed *et al.*, 2022b).

CONCLUSIONS

From this study, it is concluded that AA and CC were the most common genotypes. The allele A of *VEGF* (rs699947) can increase the risk of RA; however, only a protective role is seen in the case of allele C. There are some possible chemotherapeutic drugs in plants like beta boswellic acid, tanshinone, baicalin etc. that can be used *via* nanoparticle drug delivery to cure RA by inhibiting BTK, auto-activation of B-cells and cytokines that damage cartilage. There are some limitations of this study in that the genetic variability cannot be accessed entirely by

the polymorphism on a functional basis because RA is an autoimmune and multifactorial disease, and the genome of the Pakistani population is relatively homogenous because of the cousin marriages.

Declarations

Author Contributions. Conceptualization, N.H, M.A, M.M, A.A.N and A.S..; methodology, N.H, M.A, M.M, A.A.N and A.S software, T.A; validation, A.A.S; formal analysis, T.A.; investigation, N.H, M.A, M.M, A.A.N and A.S ; resources, M.A and A.A.S.; data curation, T.A.; writing—original draft preparation, T.A and A.S.; writing—review and editing, M.E.A, A.F.A and A.S; visualization, N.H; supervision, T.A.; project administration, A.A.S and M.A; funding acquisition, T.A

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Conflicts of Interest. The authors declare no conflict of interest.

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