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In silico investigation of inhibitory activity of Nimbaflavone, a constituent of Neem (*Azadirachta indica*) against the PTPN22 and validation of its stability by Molecular Dynamics Simulation

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Abstract

Neem plant (*Azadirachta indica*), a traditionally known medicinal plant is very effective in various diseases. We are trying to assess the activity of Nimbaflavone, one of the extracts of Neem plant in *In silico* approaches to understand the role of this extract in inhibition of intermediate molecules associated in the pathway of rheumatoid arthritis. PTPN22 has been found to be linked with the autoimmune diseases including RA, which play significant role in T cell signalling. The established function of PTPN22 in the functioning of CD8 memory T cells and the development of interleukin-17-producing T helper (Th17) cells, which participates in the production of proinflammatory cytokine. In our investigation we have focus on the inhibitory effects of Nimbaflavoneon the activity of PTPN22 and STAT4 by using the PyRx virtual Docking tools. The docking simulation study demonstrated Nibaflovone bind effectively with PTPN22 with docking score **-8.5** kcal/mol compared to positive control methotrexate. Molecular dynamics simulation study of PTPN22 & STAT4 complexed with Nimbaflavone was performed by using Discovery studio software to establish the better binding affinity and stability of

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Nimbaflavone-PTPN22 complex due to presence of sufficient no. of hydrogen bonds, hydrophobic bonds and electrostatic force of interaction. Further, *In Vitro* and *In Vivo* study need to be required in order to prove efficiency and efficacy of Nimbaflavone against PTPN22.

Key words: Nimbaflavone, PTPN22, Docking Study, Molecular Dynamic Simulation, rheumatoid arthritis, Inflammation etc.

Introduction

The various part of neem plant (*Azadirachta indica*) has been used for the therapy of several ailments such as Chickenpox, fever, headache, leprosy, jaundice, constipation, respiratory problems, rheumatism, and gastrointestinal disorders and also the important ingredients of commercial products soaps, *toothpaste*, and pest repellentstraditionally(Eid et al., 2017; Heyman et al., 2017; Saleem et al., 2018). The phytochemical analysis of the neem plant has revealed the existence of several elements, including flavonoids, catechins, anthocyanins, quercetins, saponins, tannins, limonoids, gallic acid, and other minor polyphenols. These constituents have been found to have biological activity in various experimental models(Alzohairy, 2016; Alzohairy, 2016; Heyman et al., 2017; Nagini, 2014). The utilization of numerous components derived from the neem plant has traditionally showed beneficial effects. As a result, researchers and academics have shown a significant interest in investigating the impact of these elements on the biological pathways associated with various disorders(Al Akeel et al., 2017; Deng et al., 2013; Ghonmode et al., 2013; Patel et al., 2016). Quercetin and β-sitosterol were initially identified as polyphenolic flavonoids that were extracted from freshly harvested neem leaves. These compounds have been recognized for their inherent antifungal and antibacterial properties(Govindachari et al., 1998).Neem has been historically utilized in traditional medicinal systems such as Ayurveda, Unani, and Homeopathy due to its recognized antibacterial, antimalarial, hepatoprotective, anti-inflammatory, and chemotherapeutic attributes(Schumacher et al., 2011a). The neem tree possesses a significant amount of liminoid terpenoids called azadiractoids, which are well-known for their anti-inflammatory qualities(Ruslie& Darmadi, 2020). The efficacy of neem leaf extract in reducing the production of TNF-α and IL-6 in colitis produced by DSS has been demonstrated(Ruslie& Darmadi, 2020). The magnitude of the decline was more pronounced when administered at a larger dosage.

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The modulation of molecular and genetic pathways is implicated in the initiation and advancement of cancer. Previous research has documented that neem plants and their components exhibit inhibitory properties against the proliferation of malignant cells through the control of cellular processes such as proliferation, apoptosis, tumour suppressor genes, and numerous molecular pathways(A. H. Rahmani et al., 2014).Previously well-established association between the inhibition of the NF-κβ pathway and the initiation of apoptotic cell death (Ruslie& Darmadi, 2020; Schumacher et al., 2011a) has been confirmed the role of Neem leaves extract to induce apoptosis in human leukaemia cells. Following the similar treatment, there was a down-regulation observed in the levels of anti-apoptotic proteins Bcl-xL, Bid, and XIAP(Schumacher et al., 2011b). The findings of this study demonstrated that Neem Leave Extract exhibited a gradual induction of apoptotic cell death, mostly through the activation of the mitochondrial cell death pathway.The proteins Bcl2 and Bax are significant contributors to the regulation of the apoptotic process. The genesis and progression of tumours are attributed to any modifications in the genes bcl2 and bax(A. Rahmani et al., 2012). The observation of modified gene expression has been documented in numerous tumours. The PI3K/Akt signalling pathways play a crucial role in the facilitation of tumorigenesis. Nevertheless, the suppression of PI3K/Akt signalling pathways represents a crucial mechanism in the control of tumour progression. The research experimentation was to examine the impact of leaf extract on the PI3K/Akt signalling system and apoptotic pathway in prostate cancer cell lines, specifically PC-3 along with LNCaP cell line. The findings of this investigation indicated that the leaf extract effectively causes apoptosis and hinders cell proliferation by inhibiting the PI3K/Akt pathway in both PC-3 as well as LNCaP cell lines undertaken (Gunadharini et al., 2011)

A following inquiry was undertaken to evaluate the influence of Nimbolide on apoptosis and insulin-like growth factor (IGF) signaling molecules in androgen-independent prostate cancer (PC-3) cell lines. The findings of this study indicate that Nimbolide exhibits substantial anticancer properties by promoting apoptosis and impeding cell proliferation through the PI3K/Akt pathway in PC-3 cells(Raja Singh et al., 2014).The NF-κβ transcription factor is known to have significant implications in the development and progression of cancer and its associated disorders(Sen & Baltimore, 1986). Nonetheless, the suppression of NF-κB activity plays a crucial role in the prevention of cancer initiation and advancement. A significant study was

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conducted to examine the effectiveness of bioactive phytochemicals in suppressing the activity and signalling of NF-κβ induced by radiotherapy (RT), as well as the NF-κβ-dependent regulation of cell death. The findings revealed that curcumin, leaf extract, and black raspberry extract (RSE) exhibited notable inhibition of both constitutive and RT-induced NF-κβ (Veeraraghavan et al., 2011).

Previous research has demonstrated the immunomodulatory and Anti-inflammatory properties of extracts derived from the bark and leaves, as well as the antipyretic and anti-inflammatory effects of oil seeds(Arora et al., 2011). An experimental study was conducted to assess the analgesic activity of neem seed oil on albino rats. The findings of the study indicated that neem seed oil had a noteworthy analgesic impact at doses of 1 and 2 mL/kg. Furthermore, it was observed that the analgesic action of the oil was dependent on the dosage administered(Kumar et al., 2012). The gene PTPN22 is responsible for the production of a lymphoid-specific tyrosine phosphatase known as LYP, which plays a crucial role in governing the immune response. This gene is considered a significant risk factor for a diverse array of inflammatory disorders, such as rheumatoid arthritis (RA)(Carmona & Martín, 2018). The involvement of PTPN22 has been identified in the signalling pathways associated with the autoimmune and autoinflammatory mechanisms that underlie rheumatoid arthritis (RA)(Carmona & Martín, 2018;Mustelin et al., 2019).

The discovery of a link between the PTPN22 gene and JIA represents a significant advancement in our understanding of the genetic factors influencing susceptibility to childhood-onset arthritis. This research offers compelling evidence for the role of the PTPN22 gene in the development of JIA(Hinks et al., 2005). Recent studies have provided evidence of a correlation between the PTPN22 gene and various autoimmune disorders, including rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), systemic lupus erythematosus (SLE), type 1 diabetes mellitus (DM), and autoimmune thyroid disease(Velaga et al., 2004).The regulatory role of STAT4 in maintaining the equilibrium between IL-12 and IL-23, as well as its involvement in the inflammatory processes associated with rheumatoid arthritis, is mediated by its ability to promote the differentiation of CD^{4+} T cells into Th17 and Th1 cell subsets(El-Lebedy et al., 2017; Gao et al., 2020). Several meta-analyses have demonstrated that the presence of a specific single nucleotide polymorphism (SNP) at rs7574865 in the STAT4 gene may be associated with

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an increased susceptibility to rheumatoid arthritis (RA). However, further investigation is required to validate this finding.The neem extract contains Nimbaflavone, which has been found to exhibit potential antiviral activity in In silico analysis. Additionally, in vitro studies have demonstrated its anti-inflammatory and hepatoprotective properties, among others(Ahmad et al., 2015). The bioactive in neem extract has demonstrated the anti-cancer potential on B(a)P induced murine forestomach tumourgenesis model(Gangar& Koul, 2008). In our *In Silico* study, our attempt is to find out the effect of Nimbaflavone, a constituents of Neem flowers in anti-inflammatory activity, targeting the PTPN22 and STAT4 gene, which play significant role in the autoimmune diseases such as RA.

1. Methodology

1.1.Preparation of ligand molecules

The three-dimensional (3D) conformation of Nimbaflavone (PubChem ID: 14492795), along with methotrexate (PubChem ID1: 26941)serving as the control, was obtained from PubChem database, and afterwards imported into the PyRx software. The structure was thenconverted into a PDBQT format and subjected to energy minimization.

1.2.Preparation of targets molecules

The three-dimensional (3D) structures of the protein targets PTPN22 (PDB ID: 2P6X) and STAT4 (Alpha fold: AFQ14765-F1) were obtained from the Protein Data Bank (PDB) database. The protein molecule processing was conducted using the Biovia Discovery Studio Software 2021. This involved the removal of HETATM and the addition of polar hydrogen atoms to the PTPN22 and STAT4 proteins. The PTPN22 and STAT4 proteins, which had been previously generated, were uploaded into the PyRx docking virtual tools as macromolecules. Subsequently, they were converted into PDBQT format to facilitate docking analysis.

1.3.Docking simulation study

The docking analysis of protein targets PTPN22 and STAT4 with ligand Nimbaflavonewas conducted using the Autodock Vina module in the PyRx software. The resulting docking scores were calculated in terms of Gibbs energy (Kcal/mol) keeping RMSD value zero. The docking parameters for the interaction between STAT4 and Nimbaflavone are as follows: center_x =

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7.05496928522, center_y = 5.67389611386, center_z = 0.416604579011, size_x = 137.724994633, size_y = 88.009207932, size_z = 124.742238562. Similarly, for the interaction between PTPN22 and Nimbaflavone in PyRx virtual tool, the docking parameters are: center_x = 16.4921323192, center_y = 20.569486072, center_z = 82.7703103438, size_x = 96.1259082469, size_y = 64.7879266436, size_z = 98.1849719599.

1.4.**Protein ligand-interaction analysis**

The interaction between the target proteins PTPN22 and STAT4 with ligands Nimbaflavonewas assessed using *Biovia Discovery Studio Software* 2021. Various types of bonding, including hydrogen bonds, electrostatic bond,and hydrophobic interactions, were analysed. The resulting PTPN22 and STAT4 complexes with Nimbaflavone were visualized and saved in .PDB format. The PTPN22 and STAT4 docked complexes have been further applied for the molecular dynamics simulation analysis by using *Biovia Discovery Studio software* in order to understand the stability and flexibility of the same.

2. Molecular dynamics simulation analysis

Thedocked pose structure of PTPN22 and STST4 complexed with Nimbaflavonehave been used to performed molecular dynamics simulation analysis by using Discovery Studio software 4.1 inthe solvation with water molecules. The discovery studio software 4.1 has been used for analysis of molecular dynamics based on the two methodology standard dynamics cascade and NAMD. In our study, standard dynamics cascade was used, which is completed in five steps: - minimisation-1, minimisation-2, heating, equilibration, and production. The dynamics protocol can be used for further running of molecular dynamics simulation in specific thermodynamics coordination. All setup is observed by 200 ps after environmental parameter specified. The data will be generated after the analysis of the end of production stage that took place at interval of 2ps. The energy minimisation has been done by steepest gradient approaches. Thus, the molecular dynamics simulation of protein complex was performed at default setting with 298k constant temperature and 7.4 P^H by standard dynamics cascade. The snapshot taken during the MD simulation throughout 200ps was used for the evaluation of trajectory. This snapshot was used for analysis of which amino group was connected with the stable interaction or bonding.

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The calculation was done based on the CHARMM Force field M(Brooks et al., 1983) along with SHAKE algorithm as per the protocol standard dynamics cascade.

2.1.**The dynamics trajectory analysis**

We have analysed the binding stability of molecule PTPN22, & STAT4 hits complexes by investigating the various evaluating technique such as RMSD of both backbone and side chain, RMSF of both backbone and side chain along with RMSD of staring and dynamics atomic position of ligands in PTPN22 ligands complex. The RMSD plot demonstrated the extent of change in the conformation of protein ligand complex deviated from X ray crystallographic structure, when solvated at room temperature 300k. it would be possible to analyse the how similar the conformation is, when they stabilised and whether any conformational change reported while simulation. The movement of atoms, molecules in PTPN22, & STAT4 complex is determined by keeping the temperature, pressure, and volume parameter constant for defined period of time. The molecular dynamics simulation has been performed to analyse the stability of protein complex in 200ps and results are further evaluated. Here the complete simulation time is 224ps, which includes heating, equilibrium, and production steps. The trajectory recognition with 100 frames has been produced by combining a production simulation time of 200ps along with production save results interval of 2ps. The interaction pattern of PTPN22, &STAT4 with ligands were graphically determined using Discovery studio visualisation **s**oftware **(Figure 3).**

Results and discussion

1. Molecular docking analysis by PyRx docking tool

The molecular docking analysis of Nimbaflavone has been performed using the protein targets PTPN22 (PDB ID: 2P6X) and STAT4. Docking score of PTPN22 is found to be highly negative (-8.5 kcal/mol) compared to the positive control methotrexate (-7.8kcal/mol). Docking score of Nimbaflavone with STAT4 (Table1.) has revealed significantly negative docking score (-8.1 kacal/mol) compared to positive control methotrexate (-8.5 kacal/mol). During the interaction of Nimbaflavone with STAT4 with four hydrogen bonds at amino acid residue A: LYS13:HZ1, A: LYS13:HZ3, A: GLN124:HN, A: LEU251:HN, hydrophobic bond at residue A: ALA118, A: LYS13, A: LEU251, A: HIS252, A: LYS13, A:PRO250 along with electrostatic force of interaction at amino acid residue A: GLU128:OE2 are seen represented in 2D and 3D structure

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of the same (Figure 2A & Figure B) and details of bonding interaction with amino acid residues are mentioned in **the Table 1**.The highly negative docking score of PTPN22 with Nimbaflavone is due to interacting with various amino acid in term of H-bonding, hydrophobic and electrostatic. The presence of electrostatic force of interaction at Amino acid residue A: GLU50:OE2 and hydrogen bond at amino acid residues at A: GLU50:OE2, A:PRO45:CA, A: TYR44:O along with hydrophobic bond at residues A: TYR38:C, O; LYS39: N, A: LYS39, A: LEU64, A: TYR38, A: TYR44, A:TYR66 in the binding area of PTPN22-Nimbaflavone complex, which increase its stability compared to positive control methotrexate (Figure 1B.).

Ligands	Targtes	Docking Score (kcal/mol)	H-bonding	Hydrophobic	Electrostatic
Nimbaflavone	**PTPN22**(2P6X)	-8.5	A:GLU50:OE2 A:PRO45:CA A:TYR44:O	A:TYR38:C,O; LYS39:N A:LYS39 A:LEU64 A:TYR38 A:TYR44 A:TYR66	A:GLU50:OE2
MTX	**PTPN22**(2P6X)	-7.8	B:ARG266:HH22 B:SER271:HN B:LYS32:O B:GLU50:OE2 B:SER35:CB B:PRO45:CA B:LEU64:O B:THR46:HG1	A:LYS39:CG A:PRO270 A:LYS39	
Nimbaflavone	**STAT4** **(**AFQ14765-F1**)**	-8.1	A:LYS13:HZ1 A:LYS13:HZ3 A:GLN124:HN A:LEU251:HN	A:ALA118 A:LYS13 A:LEU251 A:HIS252 A:LYS13 A:PRO250	A:GLU128:OE2
MTX	**STAT4** **(**AFQ14765-F1)	-8.5	A:GLN329:HE22 A:SER499:HG A:TRP500:HE1 A:PRO328:O A:GLY249:O		

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Table 1. Representing the interaction of Nimbaflavone with various amino acid residue in protein targets PTPN22 & STAT4 keeping methotrexate as control

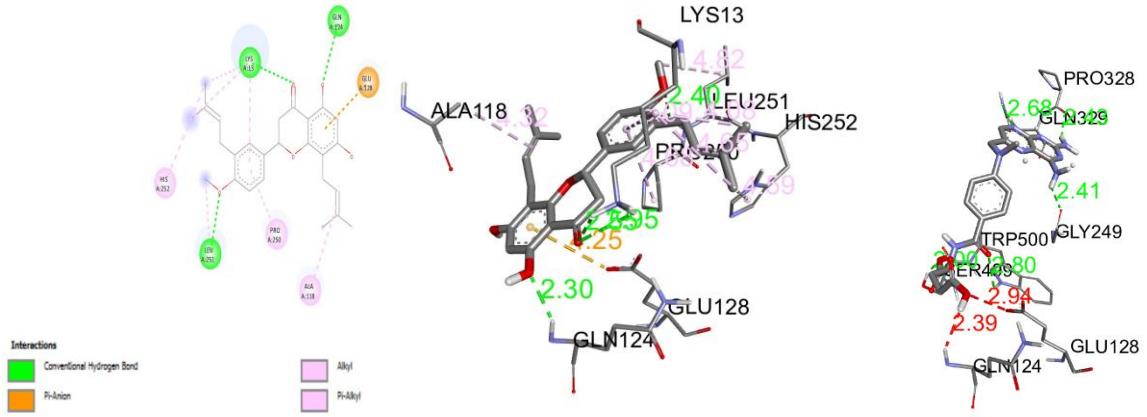

Fig: A **Fig: B** **Fig: C**

Figure1.(A-C): Representing the 2D (A) &3D (B) interaction structure of Nimbaflavone with STAT4 along with interaction with Methotrexate (C) as control

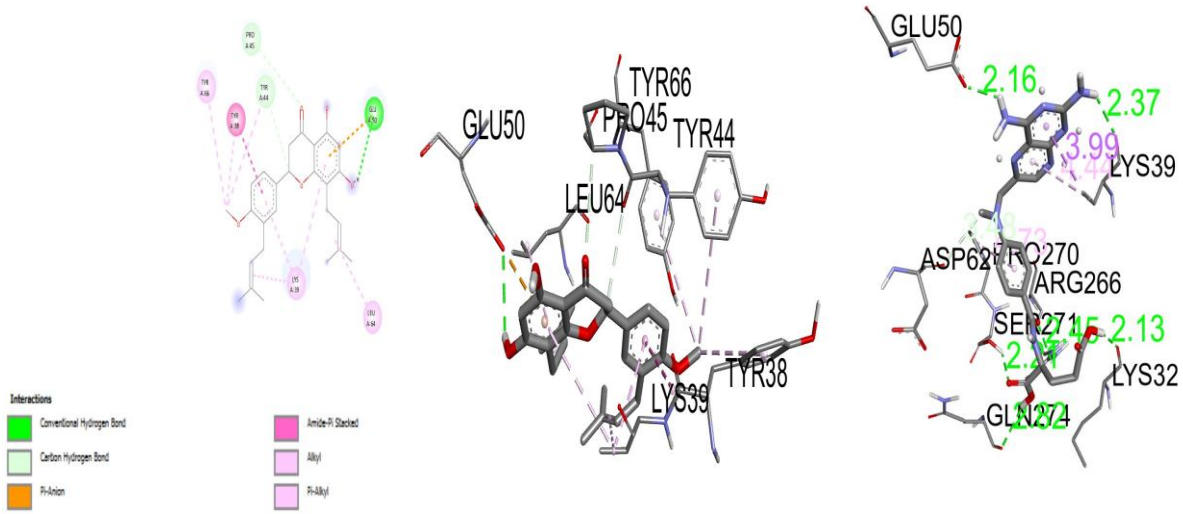

Fig: A **Fig: B** **Fig: C**

Figure 2. (A-C) representing the 2D (Fig: A) & 3D (Fig: B) interaction structure of Nimbaflavone with **PTPN22** protein and keeping methotrexate as positive control (Fig: C)

2. Molecular dynamics simulation study of phytocompounds Nimbaflavone

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A molecular docking research was conducted to investigate the interaction between the phytochemical Nimbaflavone and two target molecules, namely PTPN22 and STAT4. The results revealed that the binding energy of Nimbaflavone with PTPN22 exhibited a significantly negative value (Gibb's Energy: -8.5 kcal/mol), surpassing that of the control compound methotrexate. Further, thephytochemical **Nimbaflavone**complexes with targets PTPN22 & STAT4 have been selected for molecular dynamics simulation analysis for 200ps to verify their binding potential and stability. These complexes are solvated and minimised the energy using the descent steepest technique in water molecules.

2.1.The Root Mean Square Deviation (RMSD)

The RMSD value of STAT4-Neohesperidin dc and PTPN22- Neohesperidin dc complexes have increased dramatically till 100 conformation, however little variation recorded between conformation 28-47 and the conformation 49 to 71 respectively. The RMSD value in this regard does not stabilise the complexes up to 100 conformations trajectory whereas the backbone RMSD value of PTPN22-nibaflavone has few variations among the 100 confirmations. In summary, based on the analysis of the root mean square deviation (RMSD) values of the complexes discussed, it can be inferred that PTPN22-Nibaflavone has a greater degree of stability. This can be attributed to the little variation in conformation from 1 to 61, followed by an increase in fluctuation that remains relatively low until all 100 confirmations are completed. In contrast, the stability behaviour of STAT4-Neohesperidin dc appears to differ from that of PTPN22-Nibaflavone. The RMSD exhibited minimal variance in the PTPN22-Nimbaflavone complex, indicating a high level of stability during the 100 conformations trajectory in comparison to the STAT4 complexes.**(Figure: 3).**

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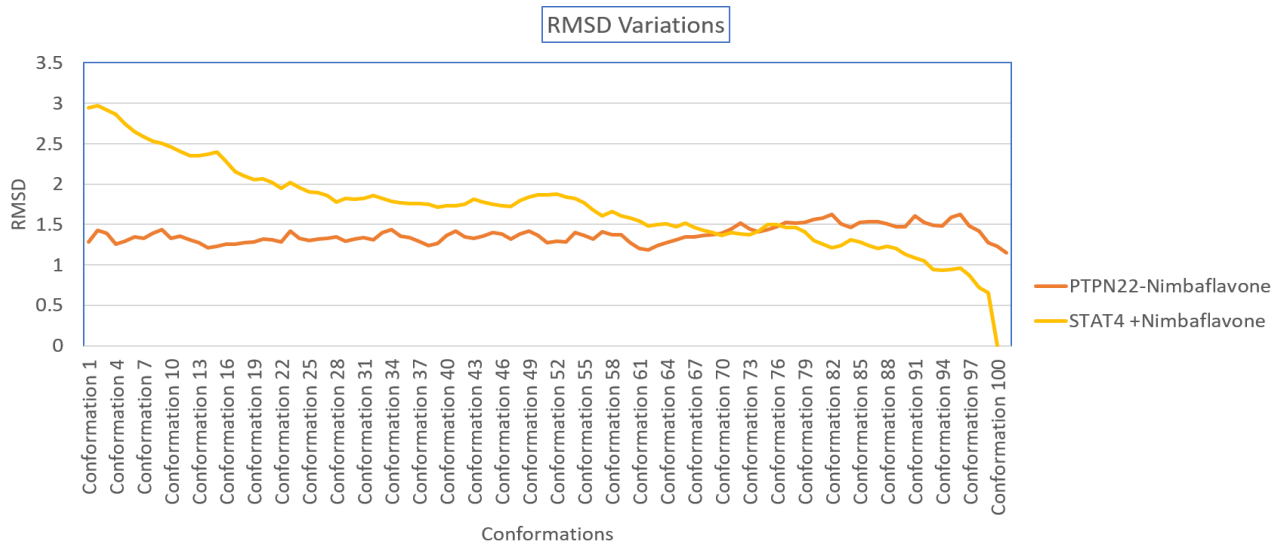

Figure 3. This graph represents the RMSD variation of 100 confirmations of Nimbaflavone complexed with PTPN22 & STAT4 demonstrating the little variation in the PTPN22 complex compared to the STAT4 complexes while MD simulation.

2.2.Root Mean Square Fluctuation (RMSF)

The fluctuation of RMSF value during the entire MD simulation run of the ligand-protein complexes is the analysis of changing the residue structural position, atom etc. over time and can also be used for the identification of maximum and minimum flexibility. The RMSF measures the extent of changing the position or displacement of atom or group of atoms compare to reference structure (Skjaerven et al., 2011; Fuglebakk et al., 2012; Martínez, 2015). The interaction of residue to the neighbouring atom or molecules may alter the extent of fluctuation in the terminal residue or surface loop region or mobility of protein.

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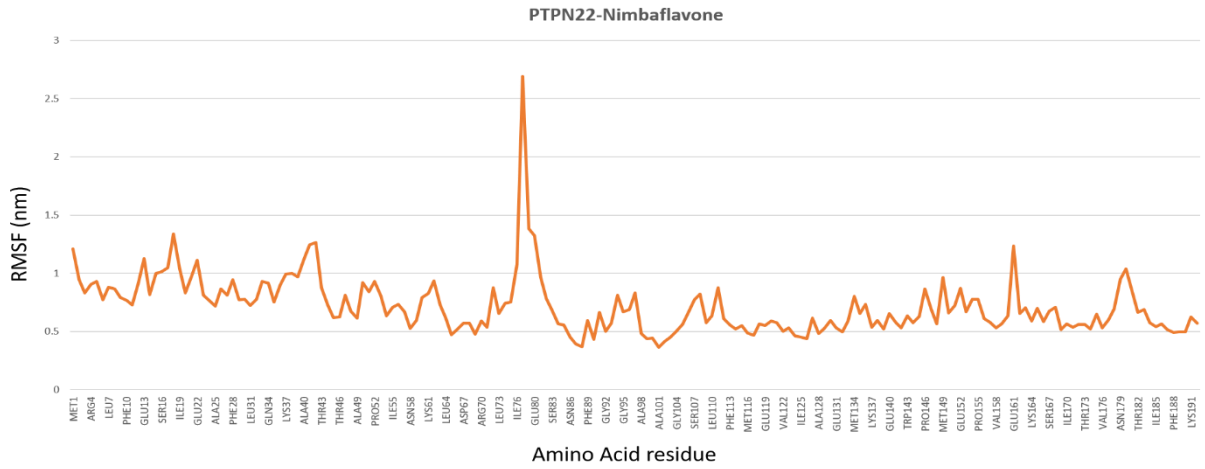
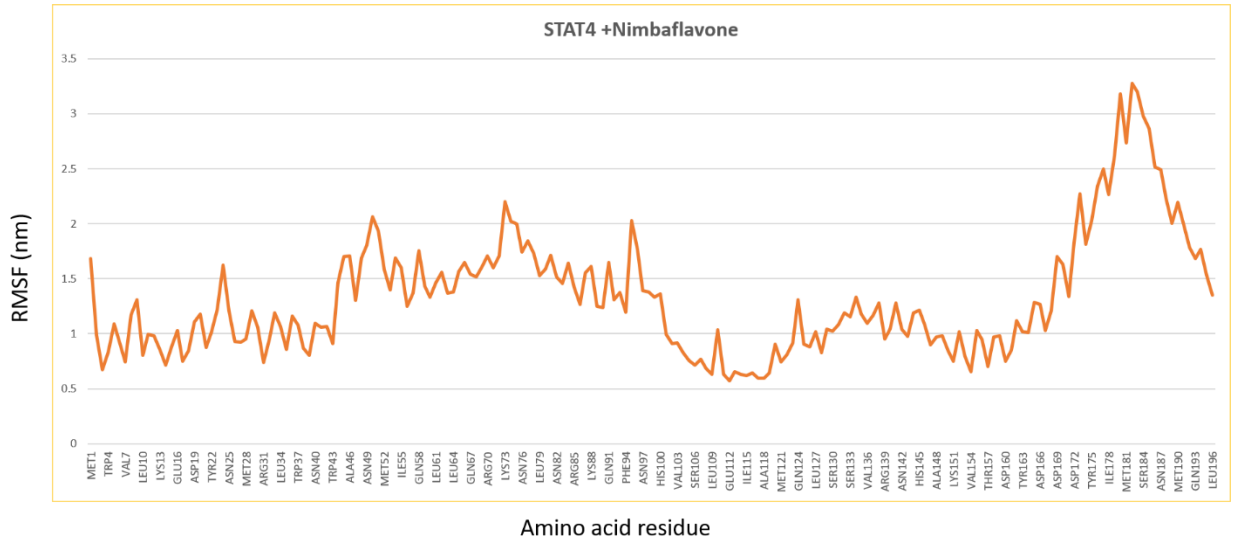


A.



B.

Figure 4 (A-B). This graph represents the RMSF variation of Nimbaflavone complexed with PTPN22& STAT4 demonstrating the little variation in the PTPN22 complex (Fig: A) compared to the STAT4 complexes (Fig: B) during entire MD simulation.

Here, the investigation of fluctuation of backbone atom or group of atoms has been carried out in the Target protein associated with KEGG pathway such as Primary immunodeficiency, catabolic pathway, extracellular degradation pathway etc. The higher RMSF value of residue in the protein complex molecules indicates the higher flexibility, decreasing the stability of complexes during the MD simulation whereas the low value shows restricted mobility with enhancing the stability of protein complexes. In our investigation of the RMSF graph, we identified PTPN22-

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Nimbaflavonecomplexes has revealed the low flexibility range from 0.25 nm to 1.25 nm and the increasing flexibility recorded from amino acid residue from ASP62-TYR94 along with the highest peak attained at amino acid THR77 residue with 2.7nm whereas the STAT4-Nimbaflavone complexes showed the higher peak at multiple position of residuesupon the completion of Molecular dynamics simulation **(Figure. A-B).**Therefore, the analysis of RMSF has demonstrated the highest flexibility of STAT4-Neohesperidin complex in our study (**Figure: 4B**) with higher RMSF value, decreasing the stability whereas the lower in the RMSF variation attributed to low flexibility, representing the comparatively high stability, which is due to presence of significant number of hydrogen bonding and non-bonding hydrophobic interaction.

3. Conclusion

According to *In Silico* techniques, the phytocompound Nimbaflavone has demonstrated the ability to block the lymphoid tyrosine phosphatase PTPN22 enzyme. This enzyme is known to be involved in various autoimmune disorders, such as rheumatoid arthritis. The presence of the C1858T single nucleotide polymorphism (SNP) inside the gene encoding protein tyrosine phosphatase non-receptor type 22 (PTPN22) results in the occurrence of a missense mutation termed R620W in the resultant PTPN22 protein(Clarke et al., 2018; Simkins et al., 2005). The presence of this genetic variant has been associated with an increased susceptibility to many inflammatory illnesses, including rheumatoid arthritis (RA). The high affinity between Nimbaflavone and PTPN22 can be attributed to the presence of specific interactions, such as three hydrogen bonds at amino acid residues A: GLU50:OE2, A:PRO45:CA, A: TYR44:O, seven hydrophobic interactions at amino acid residues A: TYR38:C,O; LYS39:N, A:LYS39, A:LEU64, A:TYR38, A:TYR44, A:TYR66, and an electrostatic interactions at position A:GLU50:OE2 with the amino acid residues of the interacting partner. The comparative analysis reveals that the flexibility of the PTPN22 complex, when bound with Nimbaflavone, is relatively reduced in comparison to the STAT4 complex. Consequently, this leads to an enhanced stability of the PTPN22-Nimbaflavone complexes. The possible improvement in the reliability of the research of inhibition can be attained by incorporating *In Vitro* and *In Vivo* experiments, alongside the utilization of molecular docking and MD techniques.

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Conflict of interest

The authors declare that they have no competing interests.

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