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Evaluation of the combined effect of naringin and imatinib in philadelphia positive chronic myeloid leukemia: An in vitro and in silico study

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Abstract---The combined effect of Naringin (NAR) and Imatinib mesylate (IMT) in Philadelphia positive chronic myeloid leukaemia was studied in vitro and in silico in this study. The chronic myeloid leukemia (CML) is a myeloproliferative hematopoietic cancer caused by a chromosomal translocation t (9;22). (q34; q11). IMT, a Tyrosine Kinase inhibitor that targets the BCR-ABL oncoprotein, is a first-line treatment for CML. IMT is related with the development of resistance due to mutation of Abl kinase domain. Natural compounds generated from plants have been linked to enhanced therapeutic efficacy and fewer adverse effects in cancer patients. Flavonoids, for example, are physiologically active substances with anti-cancer capabilities that influence a number of cellular signalling pathways suppressing a variety of malignancies. The primary goal of combining synthetic medications with phytochemicals is to achieve a therapeutic activity with the greatest pharmacological efficacy. Targeting AKT with flavonoid (NAR) compounds in conjunction with IMT treatment could be extremely beneficial in CML through additive processes. The combined effect of IMT and NAR in the treatment of CML was assessed using a silico molecular docking and long-term in vitro cytotoxicity assay on K562 cells. IMT and NAR were docked with target proteins 4GV1 and 4GV2 using the AutoDock-Vina programme. From the result of combinatorial approach, it was concluded that combining IMT and NAR improves anticancer efficacy when compared to single treatment as NAR by inhibiting carcinogenic signalling pathways

found to boost IMT's anticancer efficacy, suggesting that this could be a unique therapeutic regimen for treating Ph-positive CML.

Keywords---philadelphia positive chronic myeloid leukaemia, naringin, imatinib mesylate, tyrosine kinase inhibitor, flavonoid, efficacy.

Introduction

A genetic translocation t (9;22) (q34; q11) causes chronic myeloid leukaemia (CML), which is a myeloproliferative hematopoietic malignancy. The BCR-ABL mutant oncogene causes oncogenic addiction in CML patients. A tyrosine kinase enzyme encoded by the mutant BCR-ABL fusion gene promotes the uncontrolled proliferation of leukemic cells. A tiny Philadelphia chromosome, defined by the BCR-ABL oncogene, is produced via the genetic translocation.^[1-3] The breakpoint cluster area of the proto-oncogene tyrosine-protein kinase (BCR-ABL1) oncogenic protein with continuously increased tyrosine kinase activity is encoded by this abnormal fusion gene.^[4] BCR-ABL protein is encoded by BCR-ABL oncogene which activates several signalling pathways including JAK-STAT, MAPK/ERK, and PI3K/AKT/mTOR. AKT's involvement in the regulatory process is one of the most important.^[5]

Even when current targeted cancer therapies are used, many tumours develop resistance. Because the present targets aren't necessary for the tumor's survival, it will almost certainly develop feedback systems that trigger other pathways, keeping it alive. The remaining cancer cell is still a malignant tumor.^[6] In spite of that it contains critical oncogenes that are thought to start and perpetuate the cancer process. The occurrence of chemotherapeutic resistance is another factor in treatment failure. It's becoming obvious that cancer stem cell resiliency may be to blame for the high occurrence of treatment resistance.^[7] According to the notion of oncogene addiction, cancer cells become excessively reliant on specific oncogenes during the multistage carcinogenesis process. Oncogene mutations also appear early in the multistage process of tumour growth, possibly because they play a critical role in shaping later aspects of the aberrant circuitry in developing cancer cells. Such early critical alterations might very well arise in tumour stem cells. In such scenario, oncogene targeting is the only potential option available for effective treatment of cancer including CML.^[8, 9]

An effective (TKI's) tyrosine kinase inhibitor, Imatinib mesylate (Imatinib, IMT) is considered a first-line therapy for CML which directly aims at BCR-ABL oncoprotein. However, mutations in the Abl kinase domain cause resistance to IMT treatment by interfering directly with the drug's binding to the binding site during long-term treatment.^[10-12] A variable degree of TKI resistance have resulted because of More than 100 BCR-ABL1 single-point mutations found in IMT-resistant CML patients. A threonine to isoleucine alteration at the 315 residue (T315I) causes clinical sensitivity to imatinib, nilotinib, and dasatinib, which generates a crucial H-bond interaction with TKIs.^[13] Drug resistance encourages the second and third-line TKI drugs. But unfortunately, all TKI's experience similar affinity problems due to mutated changes in binding protein

cast down the efficacy of new TKI's in CML and IMT resistant CML cases. [14- 15] The PI3K/AKT/mTOR pathway components are becoming a beneficial target to control cell proliferation in CML patients. In CML, AKT is a critical downstream signaling molecule that is constitutively phosphorylated during apoptosis. [16, 17] Inhibition of phosphorylation/ Dephosphorylation of AKT controls cell proliferation and strongly suggests that AKT is another potential therapeutic target in the treatment of CML. Because ERK1/2 signalling is a crucial regulator of cell growth, inhibitors of the ERK pathway are being tested as potential anticancer drugs. [18]

Search for a safe and sound drug remains challenging and leads to novel therapeutic approaches. Natural products are beneficial for drug discovery because they allow scientists to identify novel active compounds that may be employed as leads or precursors in drug delivery systems with better biological characteristics. According to scientific studies and conventional knowledge, a high intake of green and other vegetables, as well as fruits, may reduce the risk of developing certain cancers. Phytochemicals and derivatives derived from plants have the potential to improve therapeutic efficacy and reduce negative effects in cancer patients. Several of these phytochemicals are biologically active compounds that have anticancer properties. [19] The plant molecules are proven effective natural sources without side effects that may act by synergy or other possible mechanisms. [20] Some of the phytochemicals have proved potential in clinical trials against CML including maytansinoids obtained from *Maytenus serrata*. Maytansinoids' anticancer properties have been related to their capacity to impair microtubule function, and they have been discovered to be potent tubulin inhibitors. [21] Similarly, Flavopiridol, derived from *Dysoxylum binectariferum*, has anti-leukemia properties. Flavopiridol has been discovered to be a broad-spectrum cytotoxic medication for acute myeloid leukaemia (AML), and it works by inhibiting cyclin-dependent kinase. It is a powerful antiproliferative drug that has been shown to have anticancer activity in clinical trials. [22]

Plants have a lot of polyphenolic chemicals like flavonoids. They are classified as flavones, flavonols, flavanones, flavanonols, and isoflavonoids, and contain two phenyl rings A, B, and a heterocyclic ring C. (often referred to as C6-C3-C6 skeleton). They are beneficial to human health and protect against cancer through an undiscovered defence mechanism. [23] Many flavonoid phytoconstituents including Oroxylin A, Quercetin, Apigenin, Kaempferol, Baicalein, Fistein, and Galangin are found to be effective against CML. [24] Similarly, Naringin (NAR) is a 4',5,7-trihydroxy flavonone-7-rhamnoglucoside found in citrus fruits and is a chief flavanone glycoside (flavonoid). [25] Antioxidant, antimicrobial, anti-inflammatory, antiapoptotic, and antimutagenic activities are the reported pharmacological activities of NAR. [26-28]. It also interacts with and affects a variety of signaling pathways. NAR has been shown to suppress a variety of cancers through modulating a variety of cellular signaling pathways, including malignant cell growth inhibition, angiogenesis, and oxidative stress mechanisms. [29] Combination therapy, specifically synthetic drugs with phytochemicals would be an innovative strategy to acquire maximum pharmacological effect. Targeting AKT with flavonoid (NAR) molecules along with IMT treatment may certainly be very useful in CML via additive mechanisms.

For cancer therapy, synergistic drug combinations seem promising and have gained a lot of interest as viable treatments for complex diseases, especially those with multiple pathogenic pathways, in the last two decades due to their effectiveness in clinical trials. However, because the mechanisms of drug synergism are widely undefined, accurate prediction of synergistic drug combinations is difficult. ^[30] Because of the large number of medications approved by the FDA, realistic tests are not feasible to examine the synergic activity of all possible drug combinations. The development of computer tools for determining the effects of medication combinations could be critical in the development of systematic screening approaches for combinatorial therapy regimens. ^[31] Among these computational methods, molecular docking is a time-saving method to predict the binding affinity and best orientation/ pose between ligand and receptor during the formation of a stable complex. The active compounds that do not fit properly in the binding site can also be recognized in molecular docking. ^[32] The molecular docking study of IMT and NAR with target protein would defiantly predict the affinity and most possible chances of the combination of these two compounds against the treatment of CML. Primary or secondary resistance to TKIs therapy still exists and still it is the first-line treatment for CML; however, there is a constant need for alternative drug delivery technologies. Considering all these research gaps in current therapy we have attempted to analyze the combined effect of IMT with NAR in Philadelphia Chromosome-Positive Chronic Myeloid Leukemia Cell Lines by using *in-silico* models and *in-vitro* cytotoxicity assays.

Materials and Methods

In- silico Molecular Docking Study

Protein Data Bank (PDB) was used to obtain the three-dimensional structures of the target proteins 4GV1 and 4QTB. PDB is an online repository for the crystallography structure of proteins and nucleic acids obtained by x-ray crystallography and NMR. ^[33] Target proteins 4GV1 and 4QTB were docked with Imatinib and phytochemical Naringin using AutoDock-Vina software. The ligands and target proteins were produced according to normal procedures, and the protein and ligand files were uploaded using MGL tools 1.5.6. The selected ligands IMT and NAR interacted with the target proteins and form a stable complex via intermolecular forces like hydrogen bonding. The binding energies/ affinity between the molecules were analyzed by Autodock tools and Pymol software.

In-vitro Cytotoxicity Assay

Reagents and Chemicals

K562 (chronic myeloid leukemia) cells were obtained as a gift sample from Dr. CD Reddy, Sugan Life Sciences, Tirupathi, Andhra Pradesh. The cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% penicillin /streptomycin, 2% L-glutamine at 5% CO₂ at 37°C. RPMI 1640 and FBS (Difco; Invitrogen Corp, Burlington, Canada); IMT, Penicillin, and Streptomycin (Novartis); NAR (Acros organics, Belgium); MTT (Sigma and Sigma-Aldrich); Cell lysate buffer (Cell Signaling Technology, Danvers, MA USA); Anti-AKT, anti-ERK,

antirabbit immunoglobulin-HRP conjugate (Santa Cruz Biotechnology, Inc, USA); Polyvinylidene fluoride (PVDF) (Millipore Corporation, Billerica, USA).

MTT Assay (Long Term In Vitro Cytotoxicity):^[34-36]

The anti-cancer efficacy of IMT and NAR was evaluated by the MTT assay method with minor modifications. The malignant cells were seeded at a density of 5.0×10^3 cells per well in 100 μ l media in 96 well plate medium on a hemocytometer and incubated overnight at 37°C. The cells were incubated for 24 hours before being treated with various doses of test chemicals. The test solution was discarded after 48 h of incubation, Plates were again incubated at 37°C for 3 hours after adding MTT solution (0.5 mg/mL). Purple formazan crystals formed at the end of the incubation time, depending on the amount of succinate dehydrogenase in the mitochondria of live cells. Each well received 50 μ l of isopropanol to help dissolve the formazan. The absorbance at 570 nm was measured after the plates were gently shaken for 1 minute. $(Y-X)/Y \times 100$ was used to compute the percentage of cytotoxicity, where Y represents the mean optical density of control (DMSO treated cells) and X represents the mean optical density of treated cells with plant extracts.

Immunoblot Analysis: ^[37]

Cells were lysed with 10 μ l of protein buffer i.e., 0.6 mol/L DTT, 10% SDS, 1 mol/L Tris-HCL (pH 6.8), 30% glycerol, 1% bromophenol blue, and boiled for 4 minutes. For 20 minutes, cell lysates were centrifuged at 16000 rpm/min. SDS-PAGE at 100 V was used to separate 50 μ g of each sample, which was then blotted onto nitrocellulose membrane overnight and blocked with 3 percent non-fat dry milk solution for half an hour to an hour at room temperature. For 1 hour at room temperature, membranes were treated with saturating concentrations of primary antibodies (Anti-AKT and anti-ERK) under gentle agitation. The gel was rinsed 3 times with TBST before being probed with a secondary antibody that was HRP-conjugated. After that, the membranes were kept at room temperature for an hour. Using Diaminobenzidine reagent(DAB), the signal was identified in X-ray film within 2-3 minutes.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 5. Multiple comparisons were assessed using one-way ANOVA-Kruskall walli's test. A statistically significant p-value of less than 0.05 was considered.

Results

IMT and NAR interacted with AKT or PKB- α Protein (PDB ID: 4GV1) and ERK1 protein (PDB ID: 4QTB) via various intermolecular interactions like hydrogen bonding, hydrophobic and non-covalent interactions. The binding affinities of NAR and IMT towards 4GVI and 4QTB and interactive amino acid residues were tabulated in Table. 1 and Table. 2 respectively. Figures 1 and 2 depicted the interactions of NAR and IMT with the active site of 4GV1 and 4QTB protein. NAR had more stability with a higher binding energy of -8.4 kcal/mol with PKB α

protein (4GV1) in comparison to IMT which has a -7.8 kcal/mol binding affinity towards it. In contrast, the IMT molecule showed a high binding affinity of -10.2 kcal/mol towards ERK1 (4QTB) whereas NAR showed -8.0 kcal/mol binding affinity upon the same.

In- Vitro Assays

Cell viability was investigated by MTT assay method. IMT was used as the standard drug of different concentrations. The IC₅₀ concentration derived via MTT assay for IMT and Nar is 5 μ M and 109 μ M respectively. NAR alone at a dose of 25 μ M concentration has a less inhibitory effect on live cells when compared with 50 μ M and 100 μ M concentrations in the MTT assay. Nine different groups were segregated and cytotoxicity of IMT at 2 μ M, 3 μ M, 4 μ M was evaluated when combined with three different concentrations of NAR (25, 50, 100 μ M) along with control (DMSO). Tabular representations of results obtained by MTT assay method were shown in Table 1. As per the results obtained, the combination of IMT (3 μ M) with NAR (100 μ M) showed maximum cytotoxic potential compared to all other tested groups in a concentration-dependent manner. Microscopical representation of Control, IMT(5 μ M), and IMT (3 μ M)+ NAR (100 μ M) were represented in Fig 3.

Immunoblot Technique

AKT and ERK proteins exhibit a vital role in maintaining the cell cycle as their inhibition downregulates the cell proliferation during cancer conditions. The cytotoxic potential of IMT (5 μ M), Naringin (109 μ M), and their combination IMT (3 μ M) + NAR (100 μ M) were analyzed by immunoblotting technique via protein expression. Fig 4A and 4B illustrated the amount of AKT and ERK protein expressed by K562 cells by mono and combined therapy of IMT and NAR. Compared to monotherapy IMT(5 μ M), IMT (3 μ M)+ NAR (100 μ M) combination also showed a similar reduction of AKT and ERK expression which demonstrates the combined efficacy of IMT with NAR against cell proliferation.

Discussion

The current research work containing *in silico* docking study and cytotoxicity study in K562 discovered that NAR has the potential to boost IMT's anticancer activity by blocking carcinogenic signalling pathways. We strongly believe that this novel combinatorial therapeutic approach would open new insights into the Philadelphia positive CML treatment. In recent years, The identification of targeted cancer medicines has progressed significantly. Even among the most powerful and impactful cancer medicines that have been approved, innate and acquired resistance mechanisms are common. These new mechanisms of resistance have been extensively investigated, allowing drug development experts to learn how such resistance could be eliminated in future generations of therapies. In some cases, novel medication candidates have been able to take the place of currently approved drugs, while others have been utilized in combination with existing therapies.^[38] Despite tremendous improvements, cancer therapy remains a deductive approach. Targeting single molecular aberrations or cancer pathways has produced great clinical outcomes with no influence on survival in select malignancies. On the other hand, using a single treatment to target a specific characteristic or route ("magic bullet") is unlikely to result in a cancer cure. It has been now very much clear that the drug combinations will be effective against a variety of molecular changes or cancer hallmarks, like what we've seen so far.^[39] A new technique for combating medication resistance resulted due to long-term monotherapy is to combine natural phytoconstituents with conventional pharmaceuticals in the treatment of cancer, increasing the therapeutic efficacy of chemotherapeutic agents at low doses, and reducing the side effects caused by high-dose anticancer drugs.^[40]

Carcinogenesis is characterized by abnormal cell proliferation and growth. Multiple downstream signaling pathways, such as PI3K/AKT, Ras/MAPK, and STAT, are upregulated in BCR-ABL oncogene expression, and their signaling is important in cancer.^[41] Phosphorylation of activated AKT causes carcinogenesis by activating or inhibiting several downstream effectors.^[42] Similarly, the MAPK Signaling Pathway is involved in cell differentiation, proliferation, and death. Activation of the MAPK pathway's ERK1/2 promotes cancer.^[43] IMT is an ERK1 inhibitor that selectively decreases the activity of Bcr-Abl in Philadelphia chromosome-positive chronic myeloid leukemia (CML).^[44] An extensive literature survey explored the cellular effect of natural flavonoids on the MAPK signaling cascade, PI3K/AKT signaling pathway, and the modulatory effect on drug resistance.^[45-46]

A computational approach i.e. docking was utilized to determine the affinity or association between two molecules like ligand-protein and protein-protein. The utilization of molecular docking has become an important part of drug development. These methods can aid in the prediction of chemical side effects. The molecular docking was performed for selected phyto constituent NAR and IMT with the target proteins PKB alpha/ AKT (PDB ID: 4GV1) and ERK1 (PDB ID: 4QTB) active site. The binding affinities of The compounds with the lowest binding affinity were chosen as hits. The binding mechanism and interaction energy of the two molecules revealed them as hits. This binding and inhibitory efficacy of NAR is supported with the same category of effectiveness with lupeol and other

phytochemicals in different studies. [47, 48] The anticancer activity of NAR in combination with IMT was evaluated by cell viability test, MTT assay. In MTT assay, MTT tetrazolium salt gets reduced to insoluble purple-colored formazan by the enzyme succinate dehydrogenase present in the mitochondria of living cells. [49] Results revealed that in comparison with various concentrations of mono agents treatment, the combination of IMT (3 μ M) + NAR (100 μ M) has maximum cytotoxic efficacy on K562 cells.

The molecular mechanism of NAR and IMT was explored by immunoblotting analysis. Antigen-antibody recognition specificity is used in immunoblotting to swiftly and reliably detect and identify proteins. Proteins, glycoproteins, and lipopolysaccharides are solubilized and electrophoresed on gels, then measured and permanently attached to nitrocellulose, PVDF, or nylon. Immunoblotting is a sensitive technique for detecting antigens that are recognised by polyclonal or monoclonal antibodies. Immunoblotting is a very sensitive method for detecting particular antigens recognized by polyclonal or monoclonal antibodies. [50] Ph-positive CML was characterized by increased expression of biomarkers ERK1 and AKT kinase. [51,52] Protein expression was analyzed after being treated with various concentrations and modes of IMT and NAR. A remarkable reduction in the expression of ERK1 and AKT was observed with IMT (3 μ M) + NAR (100 μ M) combination. As predictable, the result confirmed the increased anti-cancer efficacy of the IMT (3 μ M) + NAR (100 μ M) combination in contrast to the effect produced by IMT (5 μ M) and NAR (100 μ M) separately. The present study exposed the effectiveness of NAR and IMT combination in Ph-positive CML via supportive *in vitro* and *in silico* results which illustrated the complementary mechanism exerted by NAR with IMT.

Conclusion

The study discovered the potential advantages of combining NAR and IMT to treat Ph-positive CML by suppressing carcinogenic signaling pathways at the same time. As a result, as compared to a single treatment, the anticancer efficacy of the NAR and IMT combination was enhanced. According to the findings, the combination of NAR and IMT might be considered a novel treatment option for Ph-positive CML.

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Table. 1

Binding energy and Binding interactions of Naringin and IMT with AKT or PKB- α Protein (PDB ID: 4GV1)

4GV1	Binding energy of ligand(Kcal/mol)	H bonding	π - π interactions	Non-covalent interactions
IMT	-7.8	Gly-278 Gly-234 Ala-230	Tyr-315 Phe-442 Gly-157	Ala-177 (CH- π) Met-281(SH- π)

Naringin	-8.4	Lys-276 Asn-279	Phe-442	Phe-161(OH-pi) Met-281(SH-pi)
			Gly-157	
			Gly-159	
			Val-164	
			Thr-211	

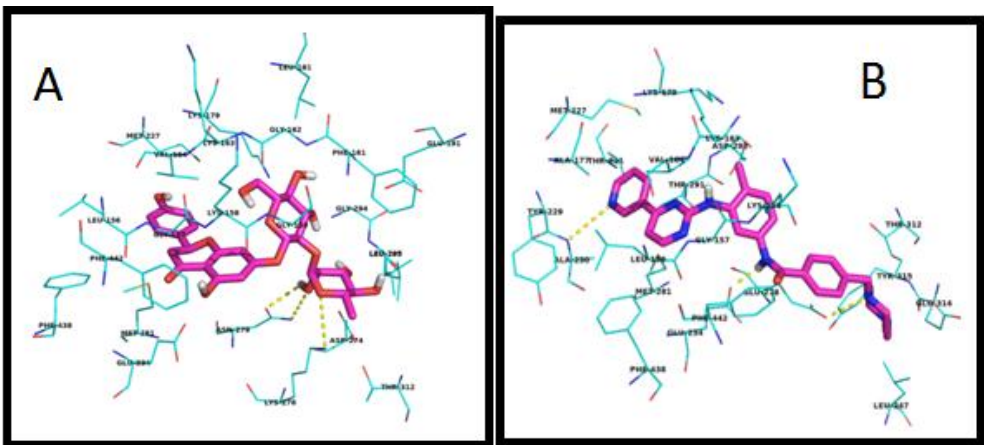


Figure 1. Molecular docking study of Naringin (A) and IMT (B) against PKB alpha (PDBID: 4GV1). Ligand molecules are shown in pink color stick style and the protein side chains and amino acid side chains are shown inline style in cyan color. Yellow color dotted lines indicated H- bond interactions with corresponding amino acid residues of the protein

Table 2
Binding energy and Binding interactions of Naringin and IMT with ERK1 protein (PDB ID: 4QTB)

4QTB	Binding energy of ligand (Kcal/mol)	H- bonding	π - π interactions	Non-covalent interactions
IMT	-10.2	-	Pro-75	Tyr-81 (OH-pi)
			Glu-88	Lys-71(NH-pi)
			Leu-173	Glu-88 (OH-pi)
			Tyr-53	Gln-122 (NH-Pi)
Naringin	-8.0	Thr-85	Phe-185	Phe-161(OH-pi) Met-281(SH-pi)
		Ile-48	Ile-101	
		Met-125	Gly-186	
		Asp-123	Tyr-53	

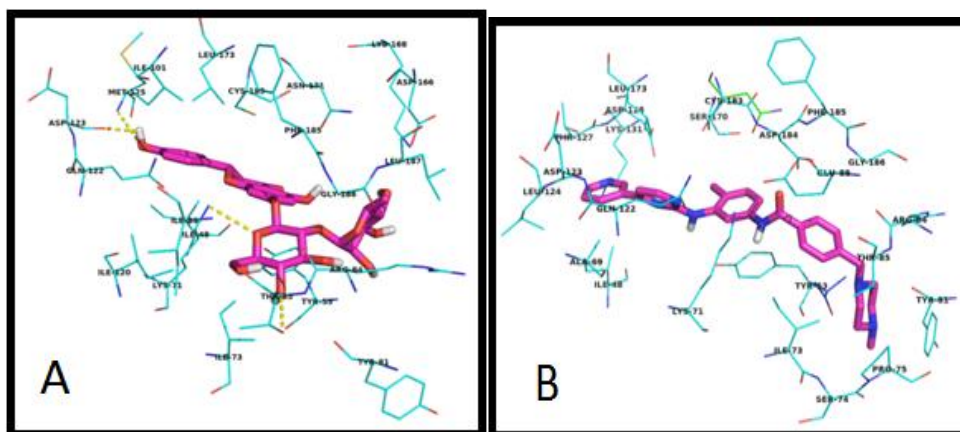
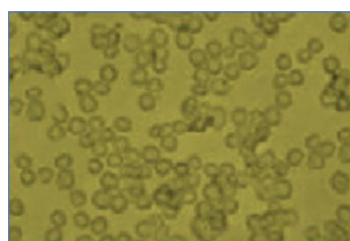


Figure 2 .Molecular docking study of NAR (A) and IMT (B) against human ERK1 (PDBID: 4QTB). Ligand molecules are shown in pink color stick style and the protein side chains and amino acid side chains are shown inline style in cyan color. Yellow color dotted lines indicated H- bond interactions with corresponding amino acid residues of the protein

Table 3

K562 cells treated with the different concentrations of IMT and NAR alone and in combination and evaluated using MTT assay

Components(μ M)	Optical density	% Inhibition	IC50
Blank	0.00	0.00	
Untreated	1.201	0.00	
Naringin			
25	0.06	94.82	
50	0.257	78.56	109.1864
100	0.537	55.23	
200	0.83	30.86	
Imatinib			
1	0.814	32.16	
2	0.709	40.94	
4	0.499	58.37	5.101
8	0.243	79.7	
Combination (Imatinib+Naringin)			
2+25	0.611	49.08	
2+50	0.476	60.29	
2+100	0.287	76.04	
3+25	0.346	71.18	
3+50	0.120	89.96	
3+100	0.019	98.37	
4+25	0.04	96.45	
4+50	0.00	No viability	
4+100	0.00	No viability	



Control

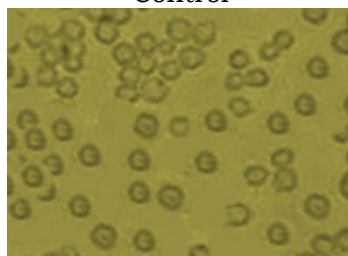
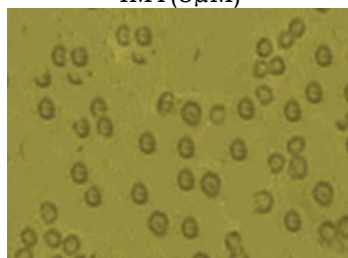
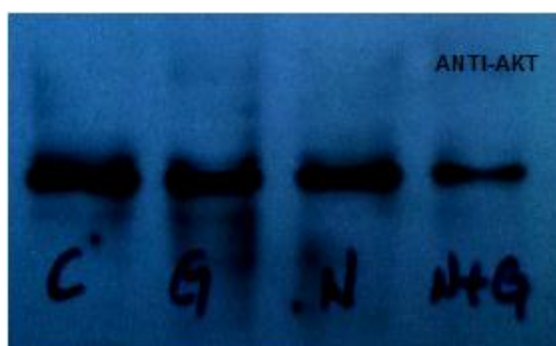
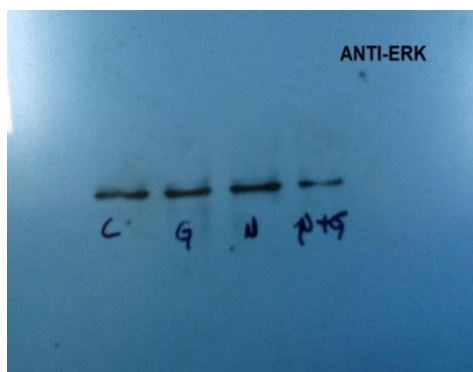
IMT(5 μ M)IMT(3 μ M) + NAR(100 μ M)

Figure 3. Photomicrographs (10X) of K562 cell lines after being treated with IMT(5 μ M) and IMT(3 μ M) + NAR(100 μ M) and control cell lines incubated for 48h



C-Control, G-(Gleevec) IMT5 μ M, N- NAR100 μ M, N+G- NAR-100 μ M+IMT 3 μ M
Figure 4A. AKT expression in K562 cells after treatment with IMT (5 μ M) and IMT (3 μ M) + NAR(100 μ M) and control.



C-Control, G-(Gleevec) IMT5 μ M, N- NAR100 μ M, N+G- NAR-100 μ M+IMT 3 μ M
Figure 4B. ERK expression in K562 cells after being treated with IMT (5 μ M) and
IMT(3 μ M) + NAR(100 μ M) and control