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In-silico Homology Modeling and Inhibitors Identification of Breast Cancer

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Abstract: ***Azadirachta Indica (Neem) is the first introduced medicinal plant in ancient medicine system, used for various disease treatments and household remedies. Lots of phytochemicals have been isolated from the various parts of the tree to study their biological activities. Gedunin has shown anticancer activity and act as Heat shock protein 90 (Hsp90) inhibitor. Breast cancer one of the most common cancer which mainly develops from lobules or ducts. Triple-negative Breast Cancer (TNBC) is an aggressive type of breast cancer in which cancer cells does not have hormone receptors. Due to lack of hormone receptors many targeted therapies were developed for advance stage cancer. The interaction between drugs and protein were examined using spectroscopic techniques, to determine the drug stability and binding site of drug in protein. Computer-aided drug design method were used to develop and analyse the drugs. Interaction between protein and drugs visualize by using molecular graphic viewer tools, to identify protein conformation and binding energy of ligand and protein.***

Keywords: Azadirachta Indica, Gedunin, Triple-Negative Breast Cancer, Targeted Therapy, Molecular Modeling, Sequence analysis.

I. INTRODUCTION

Azadirachta Indica (Neem), one of the most known and first introduced medicinal plant in siddha medicine (ancient medicine system), has been useful in treatment of various diseases. [1] Every part of neem tree has their own properties and used as household remedies since primitive times. [2] Lot of neem compounds have been isolated from various parts of the tree in which most important phytochemicals are quercetin, beta-sitosterol, azadirachtin, nimbin, nimbidin, gedunin, nimbolide, limonoids, salannin etc. [3-5] These phytochemicals are used to study their biological activities including antifungal, antibacterial, anti-inflammatory, anticancer, antiviral, antipyretic, antimalarial etc. [5] In past research, neem components has been widely used to study for its anticancer activities. Neem components activates programmed cell death, inhibit cell proliferation, inhibit cell growth and development and prohibit metastasis. [6]

Gedunin is a tetranortriterpenoid extracted from neem seed oil. It is used in various biological activities like antimalarial, antibacterial, antidiabetic and insecticidal. In recent studies, gedunin has shown the anticancer activity by inhibiting cell proliferation of cancer cell in ovary, prostate and colon. [7-8] Gedunin also act as a Heat shock protein 90 (Hsp90) inhibitor. Hsp90 is an ATP-dependent chaperone protein and initiates when cells undergo environmental pressure. It is also involved in carcinogenesis process which includes independence in growth signals, maintenance of mutant protein, growth and development of cell and metastasis. [9-10] Lamb et al was the one to discover gedunin as Hsp90 inhibitor, he also reported that gedunin inhibits an androgen receptor (AR) mediated signaling. [11] In prostate and ovary cancer, gedunin inhibit the cancer cell growth, induce apoptosis and reduce the expression of Hsp90. [8, 11] Gedunin shows cytotoxic effects in cancer cells of colon cancer. [7]

Breast cancer is one of the most common cancer which occur due to the abnormal growth of cells in breast. Most breast cancers arises from lobules or ducts. Risk of breast cancer development occur due to reproductive history, radiation therapy treatment, personal breast cancer history, genetic mutations etc. Most cases of inherited breast cancer are because of mutation in BRCA1 and BRCA2 gene. As the cancers grows from stage I to IV, stage IV breast cancer cells can spread to different body parts via blood vessels and lymph vessels. These metastasized breast cancer cells usually spread to the bone, liver, lungs and brain. [12] It is estimated that in next 10 years the number of death rate due to breast cancer will be almost 25% in comparison to current statistics. [13] Breast cancer can be classified on the basis of presence or absence of receptors in breast cancer cells. Hormone receptor-positive cancer has either progesterone receptor (PR+) or estrogen receptor (ER+) or both the receptors present in breast cancer cell.

In Hormone receptor-negative cancer, breast cancer cell does not have progesterone receptor or estrogen receptor. [14] Human epidermal growth factor receptor 2 (HER2) proteins helps in the growth of breast cancer cell. Higher level of HER2 receptor in breast cancer cell leads to HER2-positive cancer. [15] Triple-negative Breast Cancer (TNBC) is an aggressive type of breast cancer has characteristics to grow and spread faster. In TNBC, breast cancer cells lacks the expression of progesterone receptor, estrogen receptor and HER2 protein, due to the absence of these receptors hormonal therapy does not work for the treatment of TNBC. [12, 16]

Chemo remains the standard therapy for treatment of TNBC, but in recent studies many therapies were developed for advance stages. Platinum salts (Cisplatin and Carboplatin) is used as chemotherapy drugs leading to the breakage of DNA cross-linked strand which is essential for homologous recombination repair deficient cells. These drugs have shown higher pathologic complete response (pCR) rates when examined for TNBC in neoadjuvant therapy. [17-18] In some patients, TNBC is associated with germline Breast Cancer gene (BCRA) 1 or 2 mutations. For the treatment of BRCA associated breast cancer some Poly (ADP-ribose) polymerase (PARP) inhibitors (Olaparib and Talazoparib) are approved and some are still being investigated. PARP has a major role in repairing the damaged DNA and BRCA mutated cells lacks DNA repairing proteins, hence, PARP inhibitors in BRCA mutated tumors leading to the DNA single-strand break following the apoptosis. [19-21] Immune checkpoint inhibitors stops the cancer cell growth, some immunotherapy drugs (Atezolizumab and Pembrolizumab) have been studied before. This article helps to understand the role of plant-derived drugs in Triple-negative Breast Cancer treatment.

II. MATERIAL AND METHODS

Gedunin, a phytochemical isolated from azadirachta indica, which exhibit anticancer activity is used to examine its role in TNBC. We explored the interaction between gedunin and Human Serum Albumin (HSA) using spectroscopic techniques, interaction of gedunin and its 6 analogue with docking techniques. HSA is protein of our interest due to its role in drug delivery and alteration of pharmacokinetic properties of drugs. HSA is a single polypeptide chain with 585 amino acid residues. It is composed of 3 alpha-helical domain (I, II and III) and each domain further divided into two sub-domain A and B. Except drugs HSA also regulates osmotic pressure and transport various endogenous compounds. HSA has two high affinity binding sites where most of the drugs binds i.e. site I and site II located in domain IIA and IIIA. [22-23] Fluorimetric experiment was performed on a JASCO FP-8200 spectrofluorimeter. The fluorescence spectrum of protein and drug shows an emission maximum at 340nm at 280nm absorption wavelength. The analysis were carried out by titrating 100µM HSA in 3ml Tris-HCL buffer with 100µL gedunin solutions and the spectra were recorded. HSA having two major binding sites leads to the binding of site markers to these specific sites. Warfarin and Ibuprofen are the site markers for site I and site II respectively. Solution of HSA and site marker with same concentration were mixed and incubated for 5-10 min to form a proper complex. Drug molecules were added to the complex, on adding drug there is change in the emission wavelength and intensity of the molecule.

Synchronous fluorescence spectroscopy is used to analyze the conformational changes in binding pocket and protein-ligand binding polarity. Conformational changes due to protein-ligand binding leads to transformation of binding pocket affecting the nearby surrounding of HSA's fluorophores (Tryptophan and Tyrosine residues). The synchronous scan were performed with constant wavelength differences ($\Delta\lambda$) between excitation wavelength and emission wavelength for HSA-gedunin complex. Upon ligand binding, the conformational shift leads to the changes in HSA's fluorophores surrounding which can be measured when wavelength shift $\Delta\lambda$ is set at 15-20 nm for tyrosine and 60-80 nm for tryptophan. For further computational analysis, crystal structure of HSA were obtained from Protein Data Bank (PDB) in PDB format (PDB ID: 1AO6). PDB stores all the information about 3D structures of biomolecules. To analyze the protein structure, functional relationship, interactions and active sites Molecular modeling database (MMDB) were used. Constraint-based Multiple Alignment Tool (COBALT) were used to study the structure prediction and phylogenetic analysis. Protein which has been obtained from PDB were docked to remove water molecules and ions within the protein and hydrogen atoms were added at appropriate geometry groups. Using AutoDock tool, ligands were converted into PDBQT format file. Interaction between HSA and gedunin were visualize by molecular graphic viewer tool, which is used for real-time 3D structure visualization of ligand and protein and helps in identification of protein conformation and their binding affinities.

III. RESULT AND DISCUSSION

Fluorescence spectroscopy is used to analyze small ligand and protein intramolecular interaction which leads to changes in conformation, binding affinity and fluorescence quenching. Fluorescence quenching is a process in which binding of ligand causes changes in protein's fluorophore microenvironment leading to the decrease in protein fluorescence intensity. Stern-Volmer equation is used to predict the mechanism of fluorescence quenching and the relationship between protein and quencher.

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$$\frac{Log\ (Fo\ -F)}{F} = LogK_a - nLog\ [Q]$$

Above equation is stern-volmer equation, here *Fo* and *F* represents the fluorescence intensity in absence and presence of ligand, respectively. *K* is a Stern-Volmer constant, [Q] is a quencher concentration. The plot *{Fo/ (Fo-f)} vs 1/ [gedunin]* yields $F\text{-}_1$ as the intercept on y-axis and *(fK)-1* as the slope, shown in Fig. 2. Hence, the ratio of y-axis and slope gives *K*. In Fig. 1, HSA fluorescence intensity observed at 330 nm for HSA-drug system. Presuming that the observed changes in fluorescence occur due to interaction between gedunin and HSA where quenching constant can be referred as binding constant of complex formation. In Table I, the high binding constant and number of binding sites shows that even with less hydrogen bonding between HSA and gedunin, the van der waals force provides stability of drug in the cavity of HSA.

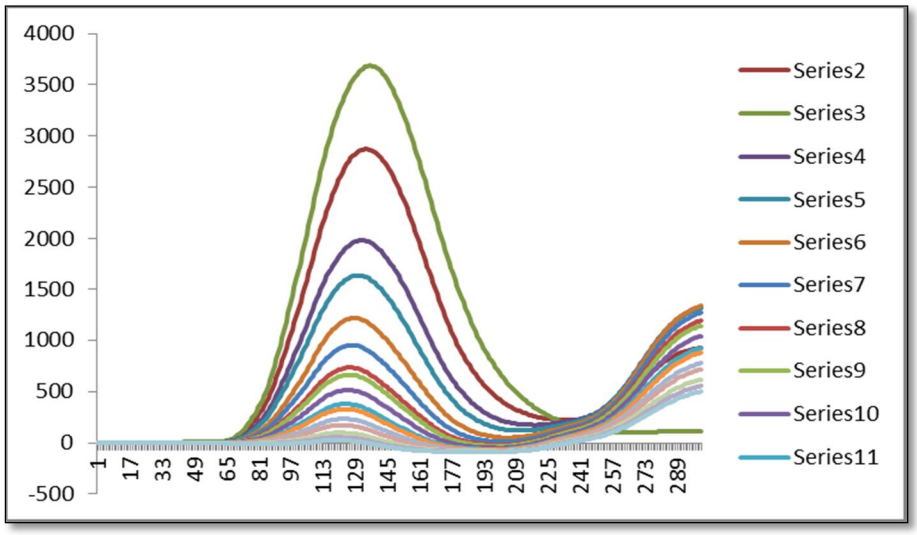


Fig 1: Spectra showing quenching of HSA's fluorescence on increasing concentration of Gedunin

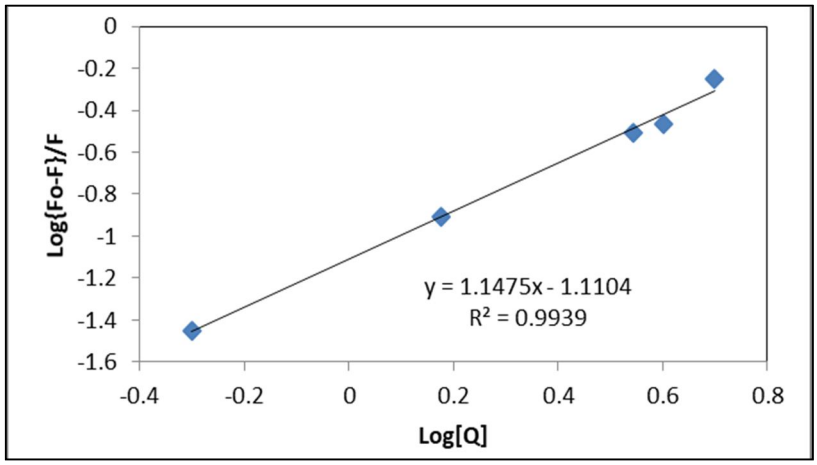


Fig. 2: Stern-Volmer plot of HSA on addition of gedunin

TABLE I
BINDING CONSTANT AND NUMBER OF BOUND PIGMENTS OF DRUG IN HSA

Sr. no.	Name	Value
1.	Binding Constant	5.975 x 105M-1
2.	Number of bound Pigment	1.1104

The synchronous fluorescence spectra for tyrosine and tryptophan were observed at Δλ= 20nm and Δλ= 80nm, respectively, shows decrease in fluorescence intensity of tyrosine and tryptophan with increasing drug concentration. Due to overlap of absorption spectrum of drug and emission spectrum of tryptophan in comparison to tyrosine enables energy transfer and location of drug in HSA regarding tryptophan and tyrosine moiety. In Fig. 3, A) shows the 3D spectra of HSA in Tris-HCL buffer and B) shows the decrease in fluorescence of HSA on the addition of drug. The stable decrease in fluorescence of both Δλ values features the conformational changes in HSA due to the binding of drug and residues.

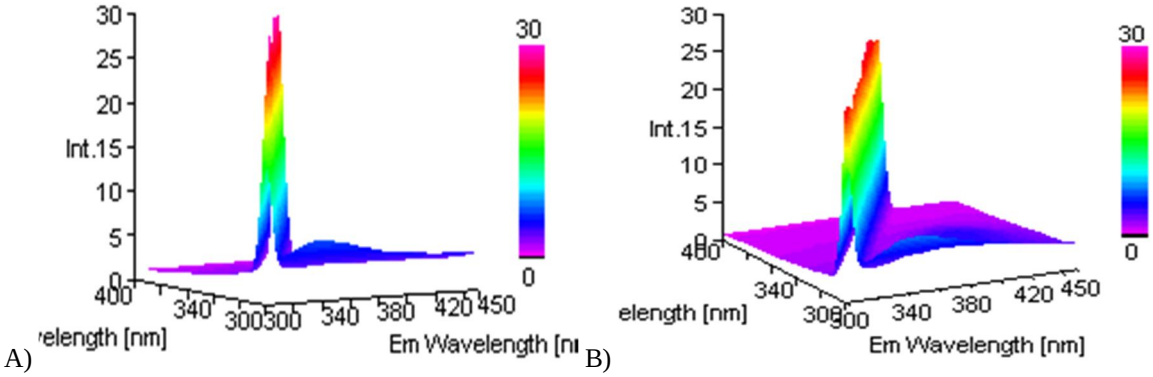


Fig. 3: A) 3D spectra of HSA and B) 3D spectra of HSA on addition of drug.

Specific site markers of site I (subdomain IIA) and site II (subdomain IIIA) are warfarin and ibuprofen, respectively. The fluorescence intensity on addition of warfarin into fluorescent HSA reduces completely and formed 1:0.5 complex. In this HSA-Warfarin complex 1µl 10mM drug is added and changes in fluorescence intensity were observed [Fig. 4]. The fluorescent intensity decreases stating that the binding of drug is in same cavity as that of warfarin and ibuprofen. Decrease in fluorescence observed on the binding of drug at site II while performing similar experiment with ibuprofen [Fig. 5]. Therefore, these results prove that the binding site of gedunin in HSA is at site I and site II.

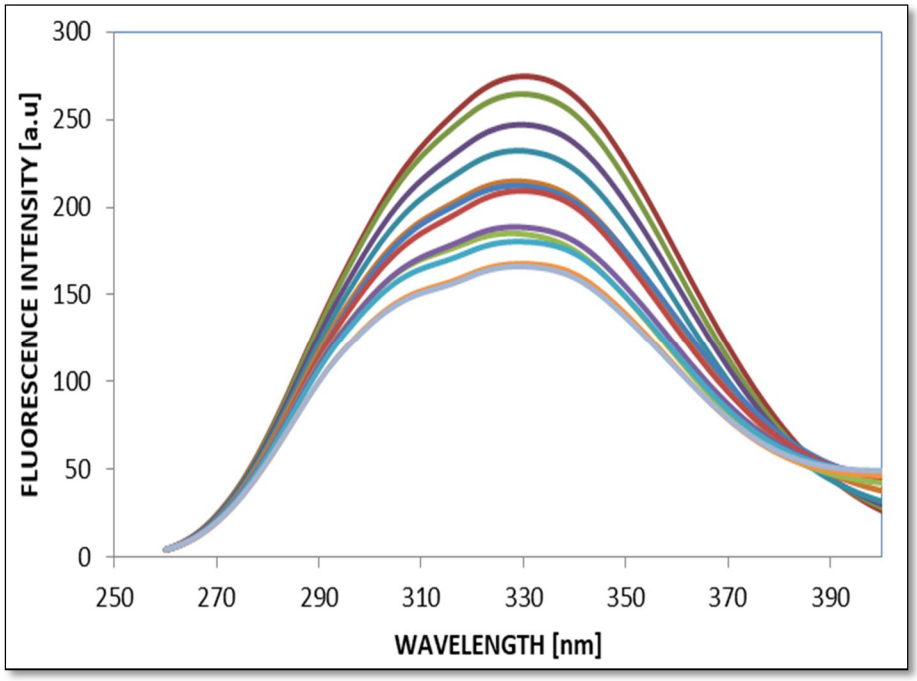


Fig. 4: Fluorescence spectra of Gedunin and Warfarin

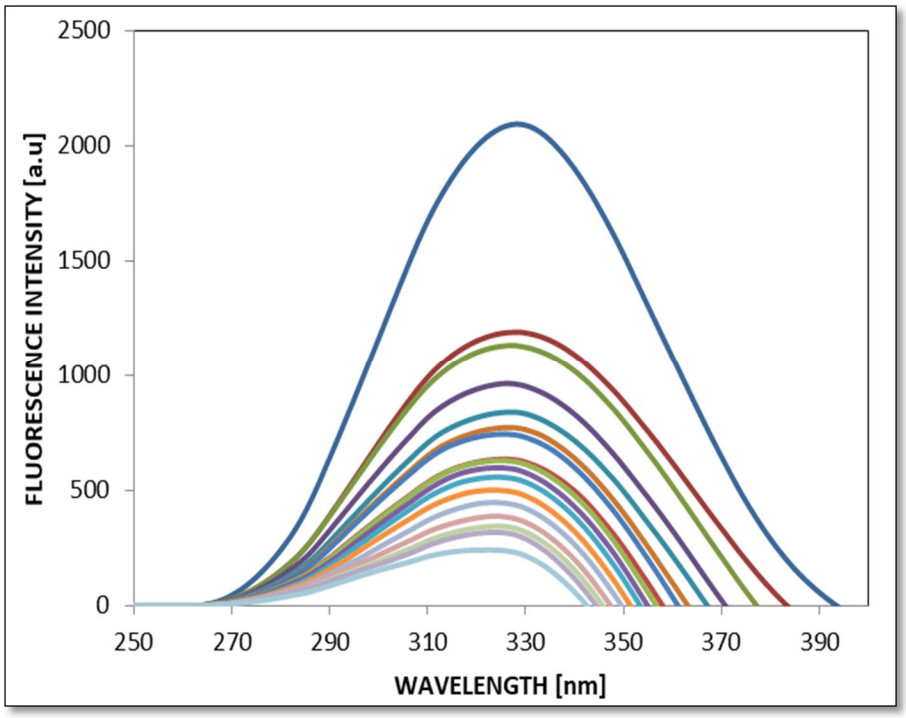


Fig. 5: Fluorescence spectra of Gedunin and ibuprofen

In Fig. 6, the graphical overview of multiple protein sequence alignment shows the sequences that matches with canonical sequence are gray in color and the sequence that are colored in red are mismatch sequence. Insertion in the sequence are represent in blue color.

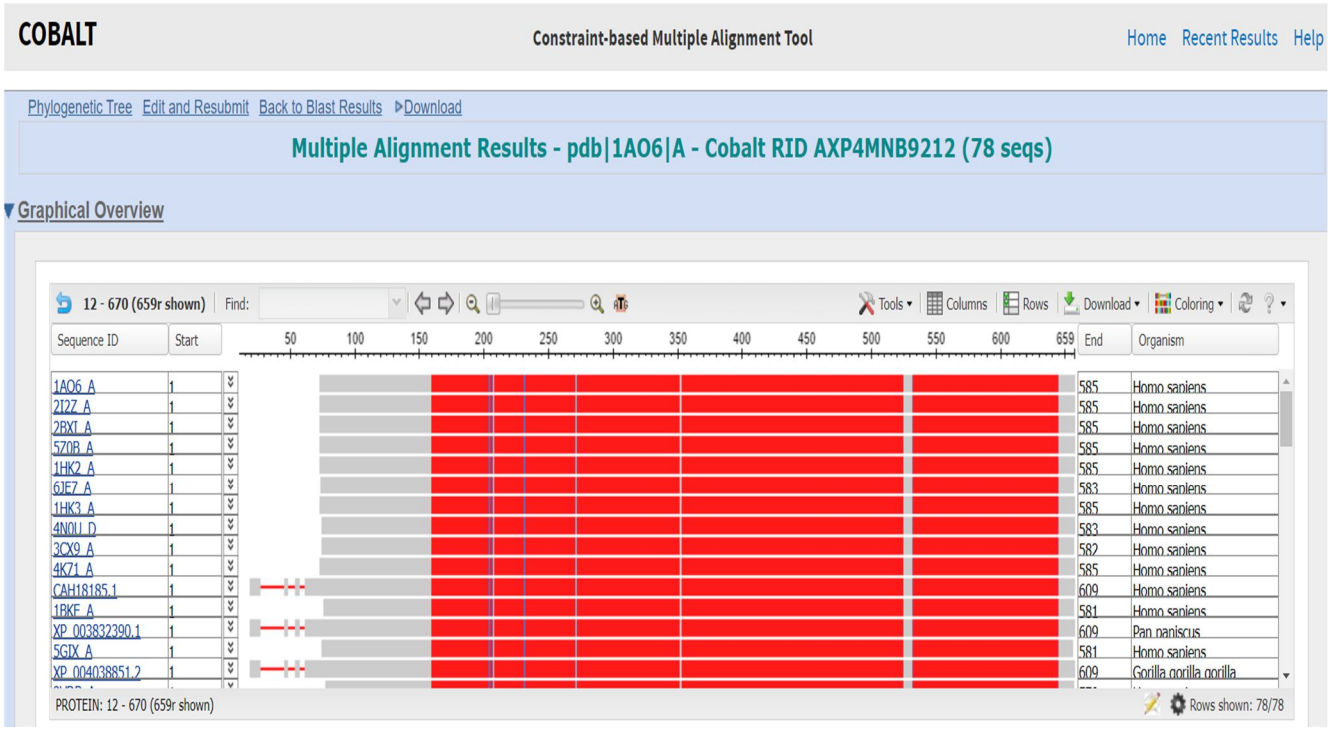


Fig. 6: Multiple alignment representation of cobalt

In Fig. 7, shapely color amino acid graph represent each amino acid residue in its given unique color.

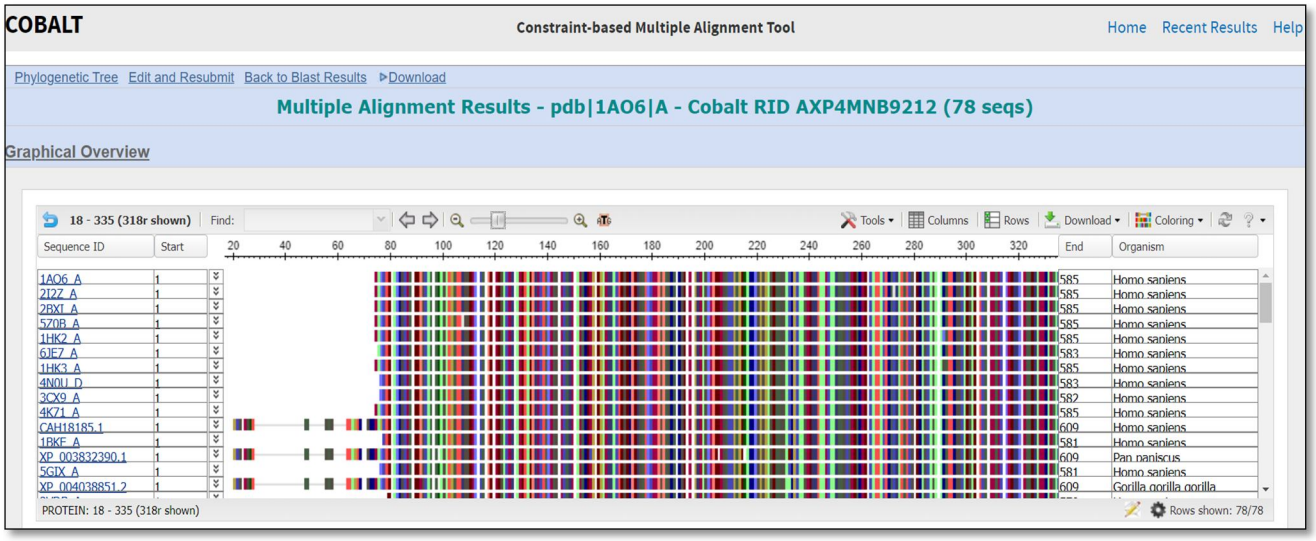


Fig. 7: Graphical overview of shapely amino acid color

To evaluate druglikeness, ADME (Absorption, Distribution, Metabolism and Excretion) analysis were performed to examine the effect of drugs in regards to safety and potency of a living organism. Gedunin and its analogue follow Lipinski's rule of 5. Bioactivity score analysis were performed against regular human receptors [Table II].

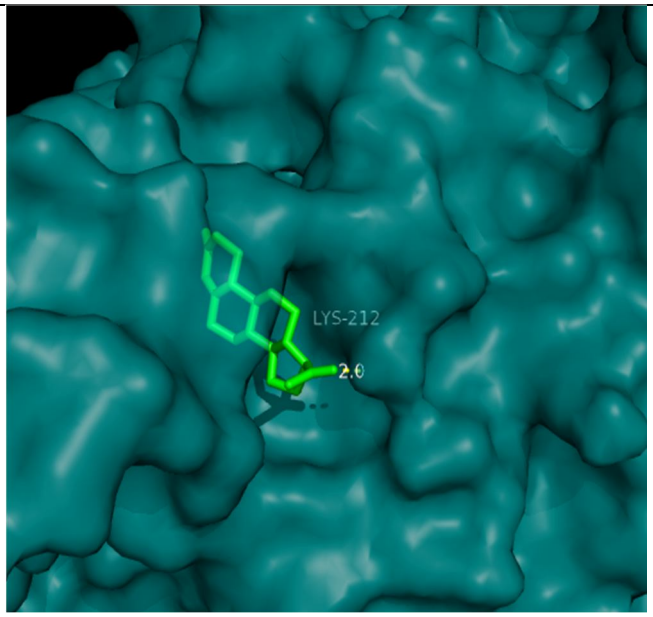
TABLE II
BIOACTIVITY SCORE OF GEDUNIN AND ITS ANALOGUE AGAINST REGULAR HUMAN RECEPTORS.

Drugs	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Parent (Gedunin)	-0.05	0.02	-0.74	0.83	-0.17	0.51
Fragment 1	-0.08	-0.38	-0.52	0.03	-0.35	0.14
Fragment 2	-0.10	-0.40	-0.52	0.03	-0.35	0.12
Fragment 3	-0.48	-0.55	-0.91	-0.21	-0.36	0.81
Fragment 4	-0.11	-0.27	-0.68	-0.04	-0.29	0.26
Fragment 5	-0.05	-0.24	-0.59	0.12	-0.31	0.16
Fragment 6	-0.00	-0.13	-0.49	0.23	-0.24	0.18

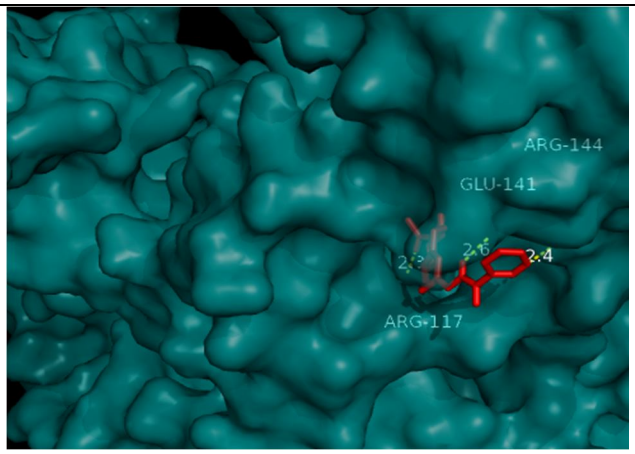
HSA consists single polypeptide chain of 585 amino acid residues and comprises 3 homologous domains: domain I (1-195 residues), domain II (196-383) and domain III (384-585). The main regions of ligand binding sites of HSA are located in hydrophobic cavities of subdomains IIA and IIIA. AutoDock 4.2 were performed to examine the binding affinity of gedunin and its analogue at the active site of HSA (PDB ID: 1AO6). 10 different conformations were generated through blind docking by AutoDock. The docking results showed that gedunin binds within the binding cavity of subdomain IIA. Gedunin and its analogue shows a binding preference for site II of HSA and the docking score below -10 shows good docking. In docking images (Fig. 8), polar residues present in domain of bound ligand specifying the polar interaction with hydrophilic groups of drugs. Protein structure shows varied nature of hydrogen bonds, bond length, binding energy and amino acid residues with different ligands are summarized in Table III. Thus, it may be concluded that gedunin and its analogues binding to a hydrophobic pocket located in the IIA sub-domain of HSA in conformity with the competitive site binding experiments.

TABLE III
DOCKING RESULT OF GEDUNIN AND ITS ANALOGUE WITH HSA

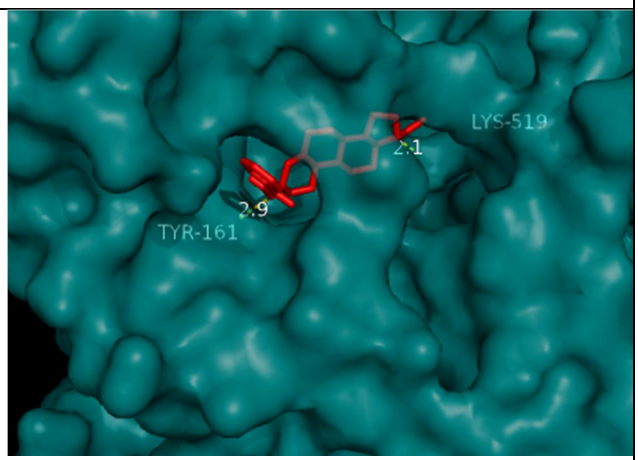
Drug	Binding energy (Kcal/mole)	No of hydrogen bonds	Amino acid residues, bond length
Parent	-9.2	1	Lys-212 (2.0Å)
Fragment 1	-9.8	3	Arg-114 (2.4Å) Arg-117 (2.3Å) Glu-141 (2.6Å)
Fragment 2	-10.1	2	Lys-519 (2.1Å) Tyr-161 (2.9Å)
Fragment 3	-9.1	1	Lys-212 (2.0Å)
Fragment 4	-9.9	1	Leu-182 (2.7Å)
Fragment 5	-10.9	1	Lys-137 (2.2Å)
Fragment 6	-11.0	1	Lys-137 (2.1Å)



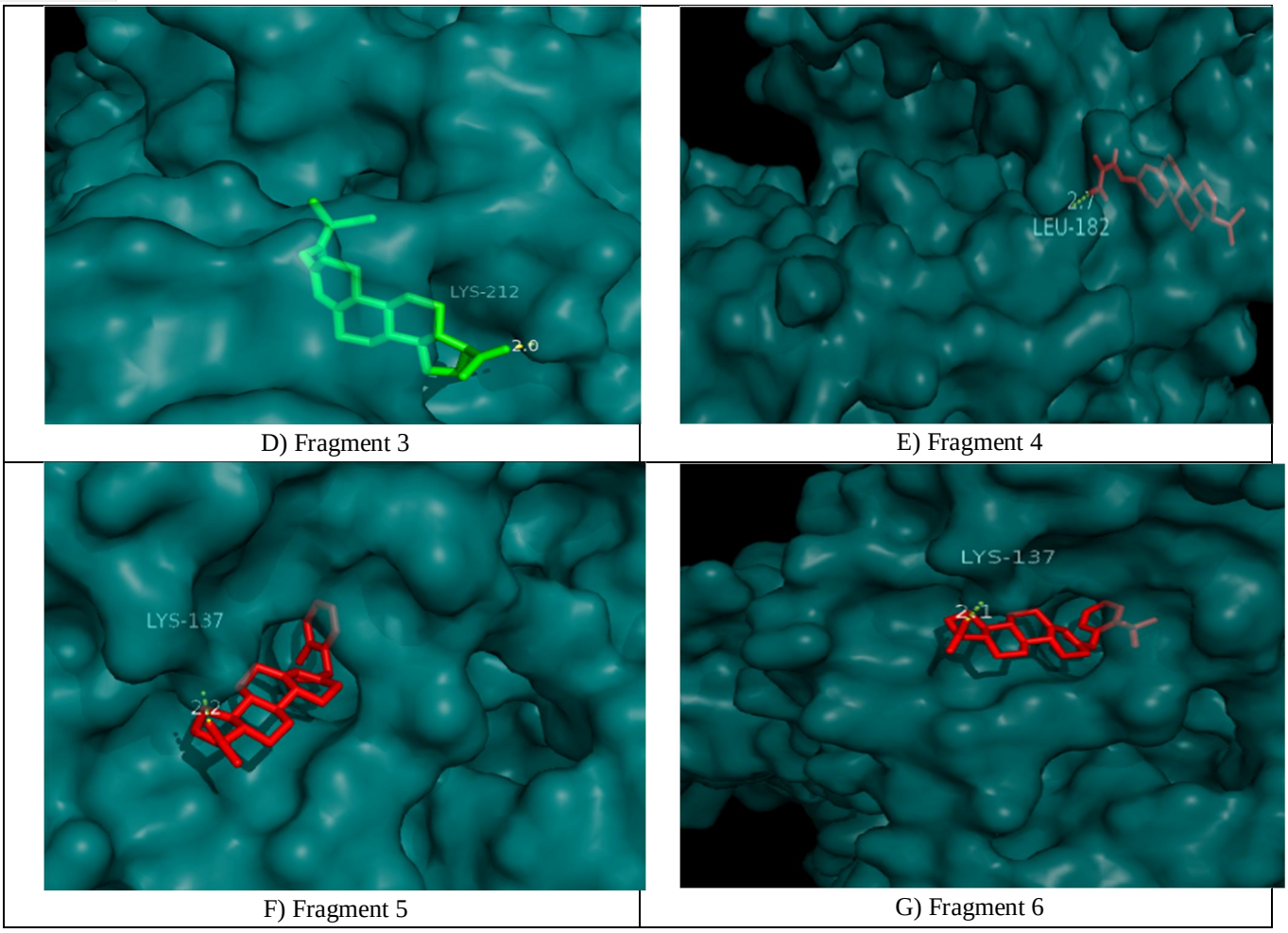
A) Parent (Gedunin)



B) Fragment 1



C) Fragment 2



D) Fragment 3 E) Fragment 4

F) Fragment 5 G) Fragment 6

Fig. 8: Docking Images of Gedunin and its analogue in the cavity of HAS

IV. CONCLUSION

In this article, Quenching mechanism determine the drug stability in the cavity of HSA provided by van der waal's force. Binding of drug with warfarin and ibuprofen proves that the binding site of drug in HSA is site I and site II. After evaluating docking results, gedunin and its analogue can be examine for further tests and analysis to provide better drug for the disease. There are so many more therapies regarding TNBC has been examined and the ongoing research for the said disease to develop an effective and safe drug for the patients. From this study we can conclude that gedunin can be useful for treatment of Triple-negative Breast Cancer. Just like gedunin we would like to continue our research for gedunin analogues to know its role with HSA binding and other activities.

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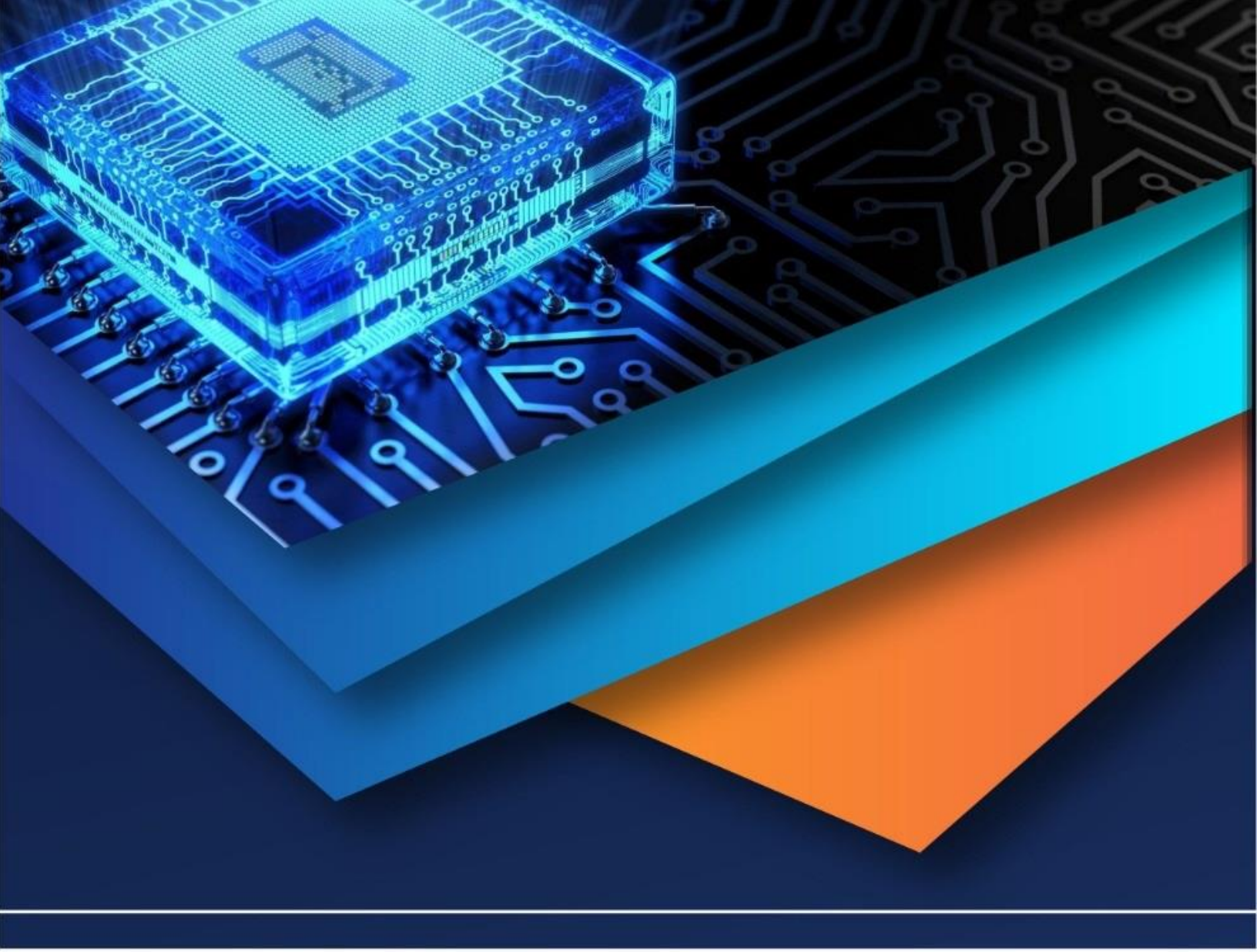

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