Anti-Cancer Activity of Phytochemical Constituents of Neolamarckia Cadamba (Roxb.) Stem Bark using Molecular Docking Studies

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Abstract:

Molecular docking studies are efficient tools to study the biopharmaceutical and pharmacokinetic parameters of drug compounds. The present study aimed to determinethe anti-cancer activity of Neolamarckia Cadamba (Roxb.) stem bark extract and to perform molecular docking on selected phytocompounds. The dried bark extracted successively by Soxhlet apparatus using n-Hexane, Dichloromethane and 80%Ethanol which was confirmed by visualization of spots on TLC using UV chamber. The extracted compounds subjected to Insilico study such as molecular docking using AUTODOCK 4.2.6 on the active sites of VEGFR2, HER2(erbB2) and EGFR Proteins. The docking results reveal among all the 3 phytoconstituents that are extracted from the Neolamarckia Cadamba bark 4-hydroxy-beta- ionone has maximum binding affinity at the active sites of HER2, VGERF2 and EGFR. The target proteins show good anti-cancer activity in response towards 4-hydroxy-beta-ionone.

Keywords- *Neolamarckia Cadamba, Molecular docking, Cancer, GC-MS, In-silico analysis.*

1.Introduction

Neolamarckia Cadamba (Roxb.) Bosser (Rubiaceae), which grows mainly in South Asia and South East Asia. The generic name owes to two Greek words, ‘Anthos’ and ‘kephalos’ indicating ball shaped flowering heads, and the species refers to its place of origin. It has been recently introduced to Costa Rica, Puerto Rico, South Africa, Surinam, Taiwan, Venezuela and other tropical and subtropical countries. It is commonly known as “kadamaba”. In folklore medicine it was used to treat fever, uterine disorders, blood related diseases, leprosy, diarrhoea, skin infections etc. Different parts of the tree were screened for antimicrobial, analgesic, antipyretic, anti-inflammatory, antidiarrhoeal, diuretic and laxative, antihepatotoxic, hypolipidemic, ant plasmodial, analgesic, antidiabetic, antioxidant, anthelmintic, antifungal, antiparasitic, ant filarial, antimalarial, antiproliferative, antioxidant, antivenom activities. However, Anticancer property not evaluated exhaustively.

Molecular Docking

Docking is a method which predicts the preferred orientation of one molecule to a second when a ligand and a target are bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to predict the strength of association binding or affinity between two molecules using, for example, scoring functions. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced (e.g., agonism vs antagonism). Therefore, docking is useful for predicting both the strength and type of signal produced. Characterization of the binding behaviour plays an important role in rational drug design as well as to elucidate fundamental biochemical processes. One can think of molecular docking as a problem of *"lock-and-key"*, in which one wants to find the correct relative orientation of the *"key"* which will open up the *"lock*. Here, the protein can be thought of as the "lock" and the ligand can be thought of as a "key". Molecular docking may be defined as an optimization problem, which would describe the "best-fit" orientation of a ligand that binds to a particular protein of interest. During the course of the docking process, the ligand and the protein adjust their conformation to achieve an overall "best-fit" and this kind of conformational adjustment resulting in the overall binding is referred to as **"induced-fit".**

Molecular docking approach:

To perform molecular docking, primarily two types of approaches are used.

I. Stimulation approach

Here the ligand and target are being separated by physical distance and then ligand is allowed to bind into groove of target after "definite times of moves" in its conformational space. The moves involve variations to the structure of ligand either internally (torsional angle rotations) or externally (rotations and translations). The ligand in every move in the conformational limit releases energy, as "Total Energy". This approach is more advantageous in the sense thatit is more compatible to accept ligand flexibility. Additionally, it is more real to assess the molecular recognition between ligand and target. However, this approach takes longer duration to estimate optimal docked conformer due to the large energy dissipating for each conformation. Recently, fast optimization method and grid-based tools have dominantlyrevolutionized this drawback to make simulation approach more user friendly. Steps involved in docking:

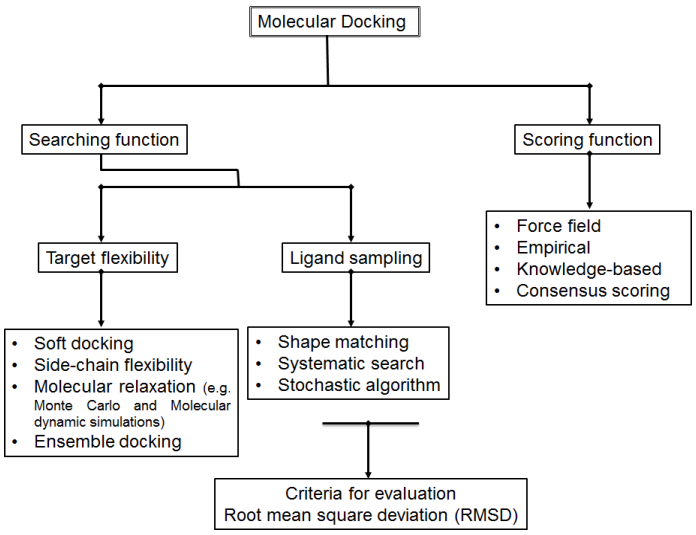


Fig 1: Molecular Docking

II. Shape complementary approach

This approach employs ligand and target as surface structural feature that provides their molecular interaction. Here the surface of target is shown with respect to its solvent- accessible surface area and ligand's molecular surface is showed in terms of matching surfaceillustration. The complementarity between two surfaces based on shape matching illustration helps in searching the complementary groove for ligand on target surface. For example, in protein target molecules, hydrophobicity is estimated by employing number of turns in the main-chain atoms. This approach is rather quick and involves the rapid scanning of numerousthousands of ligands in a few seconds to find out the possible binding properties of ligand on target molecular surface.

III. Searching function:

Comprehensively utilized docking tools employ search algorithms such as genetic algorithm, fragment-based algorithms, Monte Carlo algorithms and molecular dynamics algorithms. Besides this, there are some tools such as DOCK, GOLD, Flex X and ICM which are mainly used for high throughput docking simulations. There are various kinds of molecular docking procedures involving either ligand/target flexible or rigid based upon the objectives of docking simulations like flexible ligand docking (target as rigid molecule), rigid body docking (both the target and ligand as rigid molecules) and flexible docking (both interacting molecules as flexible).

some of the software's used in molecular docking: AUTODOCK TOOLS, PYMOL+ CONSOLE, BIOVIA DISCOVERY STUDIO.

IV. Scoring function:

The main objective of molecular docking is to attain ligand-receptor complex with optimized conformation and with the intention of possessing less binding free energy. The net predict binding free energy (ΔG bind) is revealed in terms of various parameters, hydrogen bond (ΔGh bond), electrostatic (ΔGelec), torsional free energy (ΔGtor), dispersion and repulsion (ΔG vdw), desolvation (ΔG desolv), total internal energy (Total) and unbound system's energy (ΔG unb). Therefore, good understanding of the general ethics that govern predicted binding free energy (ΔG bind) provides additional clues about the nature of various kinds of interactions leading to the molecular docking.

2.Materials and Methods:

Plant material collection and identification

Neolamarckia Cadamba stem bark was collected identified and authenticated. It was collected at Langar house, Mehdipatnam, Hyderabad, Telangana state. The collected bark dried under shade for two weeks and grounded to powder using cutting mill and stored in air tight containers.

Solvents and Reagents:

n-Hexane and Dichloromethane, precoated TLC plates purchased from Sri Venkateshwara Scientific Suppliers, Hyderabad.

Extraction:

1.0 kg of stem bark powder was extracted with n-hexane, dichloromethane and 80% alcohol successively by hot percolation method using Soxhlet apparatus. From the extracts, solvent was recovered using rotavapor, dried under vacuum and stored in a desiccator. The weight of the extracts was 6.7gm, 15.8 gm and 50.4 gm respectively. The colour and consistency of the extracts wasnoted.

Phytochemical Screening and TLC:

The phytochemical screening of all the extracts was carried out with freshly prepared reagents. Neolamarckia Cadamba Dichloromethane Extract (NCDME). The GC-MS screening is performed. Initially, the spots were observed in UV cabinet under short and longer UV light.

GC-MS analysis of the extract was performed.

In-Silico studies:

Three molecular structures have been selected from the GC MS data and their 3D structures were drawn using Marvin sketch 3D tool and the energies were minimized. These 3D structures were used for the molecular docking and to study their binding activity on three anticancer drug targets.

Docking Procedure:

2D Structures generation:

The 2D structures were generated by drawing the designed structures using an offline tool called Chemdraw, and then saving them in the formats like .cdx or. mol for further conversions of molecules.

3D Structures generation:

The 2D structures of molecules were converted into 3D structures by using PRODRG server (online tool) and Chemdraw 12.0 software. For the generated structures energy minimization was employed with 1000 iteration steps for each molecule by using PYMOL software. The generated and energy minimized 3D structures were downloaded as PDB coordinates with polar hydrogens. These PDB structures were used in the molecular docking studies.

Protein preparation:

VEGFR2 kinase (lung cancer target), HER2 (erbB2) kinase (breast cancer target) and EGFRtyrosine kinase (liver cancer target) were selected basing on the cell lines selected for cytotoxic activity. The 3D crystal structure of VEGFR2 kinase (PDB ID: 4ASD, Resolution: 2.03 Å), HER2 (ErbB2) kinase (PDB ID: 3PP0, Resolution: 2.25 Å) and EGFR tyrosine kinase (PDB ID: 4HJO, Resolution: 2.75 Å) were retrieved from protein data bank (RCSB PDB). Protein preparation was performed by removing the protein complexes using the PYMOL software and the protein molecule and cocrystal were saved separately as a PDBfile. These PDB structures of the proteins were refined by removing water molecules and adding Kollman charges by using AUTODOCK software,
then prepared protein saved as a PDBQTs file, that was used in the Grid file generation for docking study. The step-wise procedure for protein preparation in auto dock is as follows.

Download the protein from the protein data Bank for example 3pp0

This is in PDB format Open this protein PDB in pymol.

Find the chains and the ligands

Now try to remove the ligands from the protein downloaded from PDB and try to savethe protein with the empty binding site and the ligand separately.
Now open auto dock.

Generate PDBQT format of protein PDB in the following way

File> read molecule> now open the PDB of protein (which was generated earlier inpymol) Delete water molecules and add polar hydrogens as following Edit> delete watermolecules Edit>hydrogens> add>polar hydrogens> press ok
Now add Kollman charges as following Edit>charges> add Kollman charges

Now save the file of protein in PDBQT format Grid >macromolecule> choose macromolecule> select the protein> windows pop out and click ok Now save it as PDBQT Files> save>PDBQT>windows pop out click on sort notes>ok Now the proteinPDBQT saved.

Ligand preparation:

The PDB structures of inhibitors were opened in AUTODOCK 4.2.6. Gasteiger charges were added to the ligand and maximum 5 members of active torsions were set for each ligand using AUTODOCK 4.2.6 tool. These refined structures were saved as PDBQTs for further docking studies.
Open ligand auto dock File> read molecule> select the ligand and then click open> ligand PDBQT opened.
Ligand> input> choose> select the ligand> click the select molecule for auto dock

Ligand>torsion tree> choose root

Ligand>torsion tree> detect root

Ligand>torsion tree> choose torsions> click on done

Ligand>torsiontree> choose no of torsions> click on dismiss after selecting no of torsions.

Ligand> output> save as PDBQT> locate the path> save

Grid parameters generation:

The grid parameters were assigned based on the knowledge of the interacting Ligand in the appropriate protein complex. Grid box was generated using grid coordinates for the active site of 3pp0 and grid points.
Generation of GPF

Grid> macromolecule> select the protein which is saved as PDBQT>

Grid> map types> open ligand> select and open it

Grid> map types> choose ligand > click the select molecule for auto dock

Grid > grid box> apply the coordinates the active site> now select the option save in currently present in file
File> output grid dimensions file> name.txt file>save>the txt file was saved this file iscalled as configuration file.

Table 1: Grid coordinates for docking

Protein With PDB ID	x-co-ordinate	y-co-ordinate	z-co-ordinate
3PP0	21.408	31.291	14.679
4ASG	-21.951	-1.151	-3.701
4HJO	24.863	19.264	7.369

Assigning docking parameters:

AUTODOCK 4.2.6 with Lamarckian Genetic Algorithm (LGA) was used for protein rigid ligand flexible docking calculations. The maximum number of energy evaluations before the termination of LGA run was 250000 and the maximum number of generations was 27000 for each ligand. Total numbers of GA runs were set to 10 and other docking parameters were set to the software default values. All the assigned docking parameters were saved as DPF file which is further used in AUTODOCK run.

Docking:

Docking of selected extracted phytochemicals with respective proteins was carried out by using molecular docking software AUTODOCK 4.2.6. In the docking the GPF and DPF fileswere run in terminal window to generate GLG and DLG log files of the respective ligands.
After docking the ligands were ranked according to their docking energy as implemented inthe AUTODOCK4.2.6. The binding energies of docked conformation are presented in table.

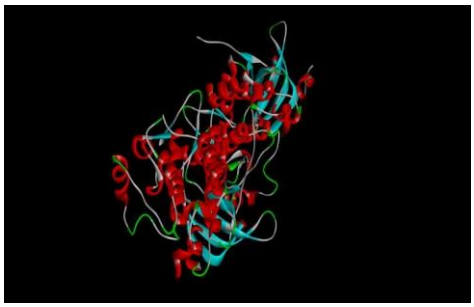
Docking analysis:

After docking the protein ligand complex of least binding conformation of each docked ligand for the target protein was built in AUTODOCK4.2.6 and taken for further docking analysis using PYMOL and even in AUTODOCK4.2.6. In this analysis, crucial interactions of selected ligands with target protein were studied and potential binding sites in ligands wereidentified by using AUTODOCK4.2.6 and 2D interactions by using Discovery studio. The step wise procedure to run DLG and find binding energies and possible interactions is as follows. analyse>see interactions> now we can see the ligand interactions in the active site of
the protein along with the amino acids with which the ligand is interacting. There must be avalid procedure to run the docking process without which we could not say whether our results predicted are correct or not, so the validation of the docking procedure is as follows.

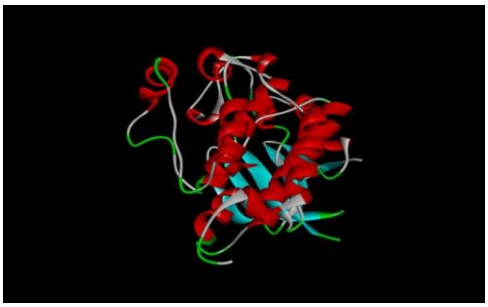
Docking validation:

In the present study, prior to the docking of designed molecules the docking procedure wasvalidated by using standard drugs SYR, Sorafenib, Erlotinib with the appropriate protein targets HER2, VEGRF2, EGFR2 respectively.
The docking conformations of the selected extracted phytochemicals and confirmations of standard drugs were superimposed to check the validity of the docking procedure. Open boththe PDBQT complexes. Observe the amount of overlapping which determines the validation of the docking procedure for that selected protein.

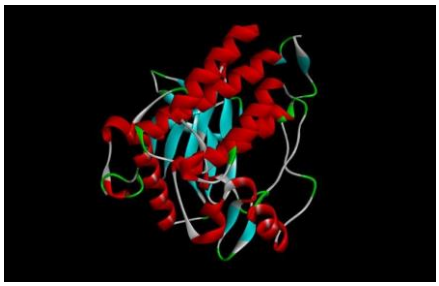
fig2: PDB format of protein:



PDB I'D 3PP0

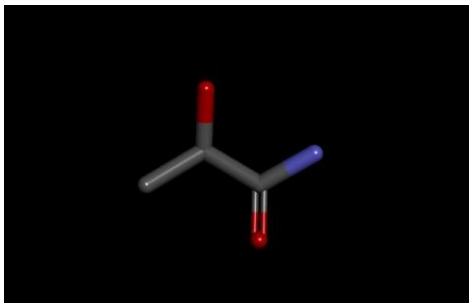


PDB I'D 4ASD

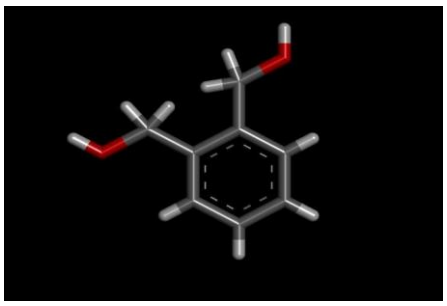


PDB I'D 4HJO

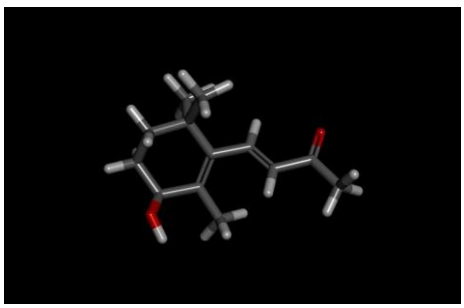
Fig 3:PDB format of phytocompounds



PDB of compound 2-hydroxypropanamide



PDB I'D 1,2 Benzenedi-methanol



PDB I'D 4-Hydroxy-beta-ionone

3. Results and Discussion

The dried bark of Neolamarckia Cadamba extracted successively by Soxhlet apparatus using n-Hexane, Dichloromethane and 80%Ethanol. Phytochemical screening was done for allextracts. Dichloromethane extract evaluated for anticancer potential on MCF7, A549 and HepG2 cell lines and subjected to GC-MS analysis. 3 phytocompounds are selected from GCMS report and studied against VEGFR2, HER2(erbB2) and EGFR tyrosine kinase through molecular docking studies

Docking Results

Molecular Docking studies:

Molecular docking was employed to study the binding patterns of the extracted derivatives with the appropriate target proteins VEGFR2, HER2 and EGFR. In order validate the docking methodology used in the docking studies, the inbuilt ligand bound to the target proteins were removed. The docked (low energy) and com-crystallized conformations were further superimposed to check their conformational relevance. The current docking procedure followed in the present study, re-produced the conformation almost equal to the co crystallized conformation of the ligands. Later, all the designed molecules were docked with the target protein following the above-mentioned validated procedure. The binding affinity and interaction of the each of the ligand was studied considering the least energy conformation of the ligand. After completion of the docking, the docking results were extracted from the appropriate dlg file.

For a molecule to act on target, there is need for the presence of some interactions of designed molecules in the target binding site. Then we can expect some bioactivity. For the prediction designed molecules bioactivity, need some standard reference molecules, here it is SYR for HER2(PDB ID:3PP0), sorafenib for VEGFR2(PDB ID:4ASD), erlotinib for EGFR (PDB ID:4HJO). The synthesized compounds showed similar interactions as standard reference and can expect similar bioactivity (anticancer), the interactions and their binding energies of the standards as well as the extracted phytocompounds and, there by the bioactivity predictions are as written in the table. The obtained docking energy of the best conformation was -7.67kcal/mol, -6.40, -5.98kcal/mol for SYR, sorafenib, erlotinib with HER2, VEGFR2, EGFR respectively. The main residues involved in the interactions with the active site of HER2, VEGFR2, EGFR were with 4-hydroxy-beta-ionone.

HER2:

The molecular docking binding energies and interactions of standard reference drug isperformed and results are,

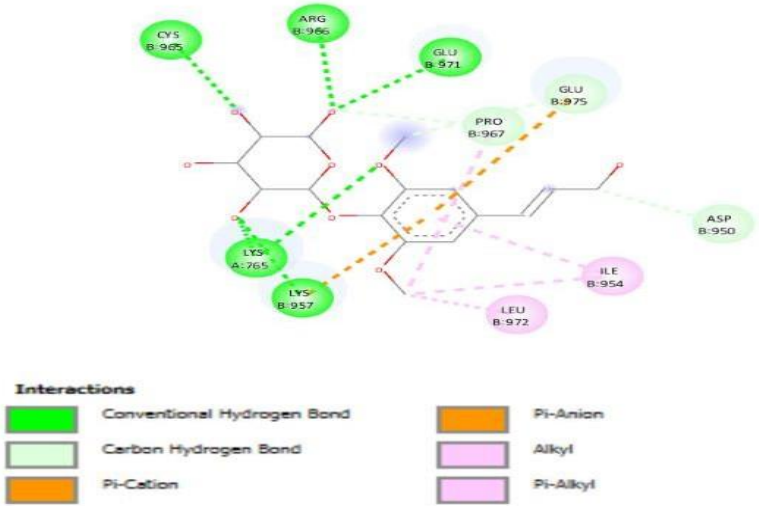


Fig 4: A.A Interaction With SYR

Table 2: Interaction of SYR with HER2

Index	Residues	A. A
1	966	ARG
2	950	ASP
3	965	CYS
4	971	GLU
5	975	GLU
6	954	ILE
7	972	LEU
8	765	LYS
9	957	LYS
10	967	PRO

All the chosen extracted phytochemicals were docked as per the above-described procedure. The binding energies of best binding pose for each were recorded and interaction with the site was studied. The results were compared with the standard drug. The binding energies of docked ligand are presented in table 3:

Table 3: binding energies of docked ligand

Ligand name	Binding energies	H-bond
SYR	-6.83	present
Propanamide,2hydroxy	-4.42	present
1,2 benzenedimethanol	-4.61	present
4-hydroxy-beta-ionone	-7.67	present

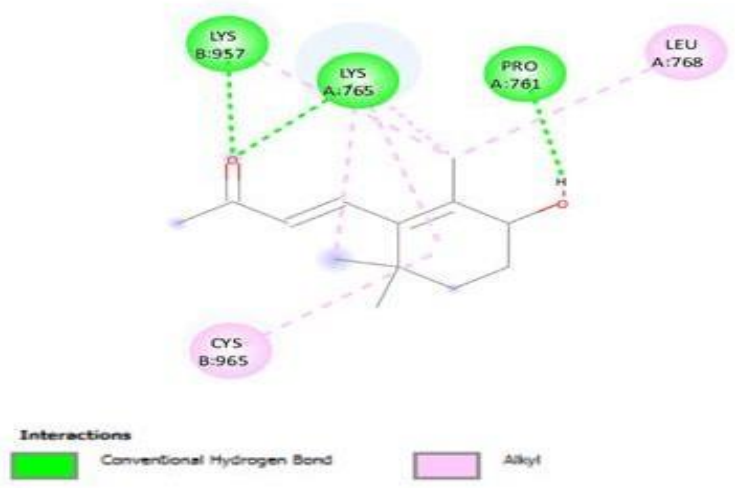


Figure 5: interaction of 4-hydroxy-beta-ionone with HER2

Interaction of extracted phytochemicals with HER2

Detailed interactions were studied for each ligand: The docking analysis of4-hydroxy-beta-ionone in the active site of HER2 (PDB ID: 3PP0) protein revealed that it has good binding with the active site, with binding energies as shown in the table with standard reference SYR has -7.67 Kcal/mol as its binding energy. The compounds showed similar interactions as that of the standard (fig) which mainly involve convectional hydrogen interactions with LYS765, LYS957were observed which can be expected to show the desirable activity.Docking analysis of extracted phytochemicals with HER2:
The docking analysis of designed extraction with the active site of the HER2 revealed that it has a good binding with the active site with binding energy -4 &-8 kcal/mol whereas the reference standard SYR, -6.83cal/mol as it's binding energy. Interactions with other extracts with amino acids were noted down in the below table 4:

Table 4: Interactions of phytochemicals with HER2

Extracted Phytochemicals	Interactions
2 hydroxy, propanamide	ASN764, LEU790, ASP962, ILE961, VAL794
1,2benzenedimethanol	TRY772, ASP769, LYS957
4-hydroxy-beta-ionone	LYS957, LYS765, PRO761, LEU768, CYS965

VEGFR2:

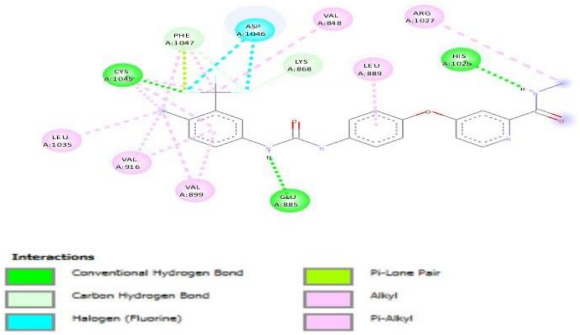


Figure 6: A.A interactions with SORAFENIB

Table 5: Interactions of SORAFENIB With VEGFR2

Index	Residues	A. A
1	1027	ARG
2	1046	ASP
3	1045	CYS
4	885	GLU
5	1026	HIS
6	889	LEU
7	1035	LEU
8	868	LYS
9	1047	PHE
10	848	VAL
11	889	VAL
12	916	VAL

All the chosen extracted phytochemicals were docked as per the above-described procedure. The binding energies of best binding pose for each were recorded and interaction with the sitewas studied. The results were compared with the standard drug. The binding energies of docked ligand are presented in table 6.

Table 6: Binding energies of phytocompounds

Ligand name	Binding energies	H-bonds
sorafenib	-7.42	Present
Propanamide,2hydroxy	-4.32	Present
1,2 benzenedimethanol	-4.14	Present
4-hydroxy-beta-ionone	-6.40	present

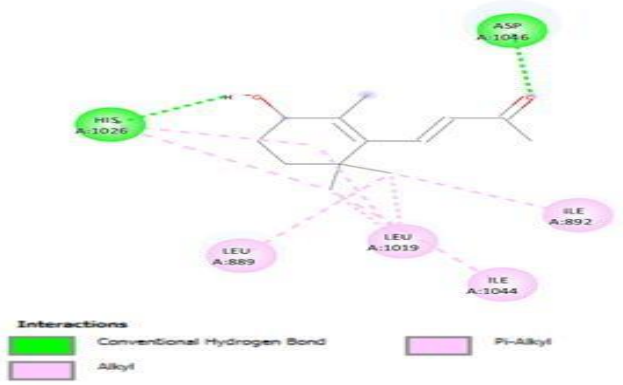


Figure 7: Interactions of 4-hydroxy-beta-onone with VEGFR2

Interaction of extracted phytochemicals with VEGFR2:

Detailed interactions were studied for each ligand: The docking analysis of 4-hydroxy-beta-ionone in the active site of VEGFR2 (PDB ID: 4ASD) protein revealed that it has good binding with the active site, with binding energies as shown in the table with standard reference SORAFENIB has -6.40 Kcal/mol as its binding energy. The compounds showed similar interactions as that of the standard (fig) which mainly involve convectional hydrogen interactions with HIS1026 and pi-alkyl interactions with LEU899 were observed which canbe expected to show the desirable activity.

Docking analysis of extracted phytochemicals with VEGFR2:

The docking analysis of extracted phytochemicals with the active site of the VEGFR2 revealed that it has a good binding with the active site with binding energy -4 &-7 kcal/mol whereas the reference standard SORAFENIB has, -7.42kcal/mol as it's binding energy. Interactions of other extracted phytochemicals with amino acids were noted down in the below table 7.

Table 7: Interaction of phytochemicals with VGEFR2

Extracted phytochemicals	Interactions
2 propanamide, hydroxy	THR1076, SER1090, LEU1029, ALA2073, TYR1082
1,2benzenedimethanol	ASP1046, HIS1026
4-hydroxybeta-ionone	ASP1046, HIS1026, LEU889, LEU1019, ILE1O44, ILE892

EGFR:

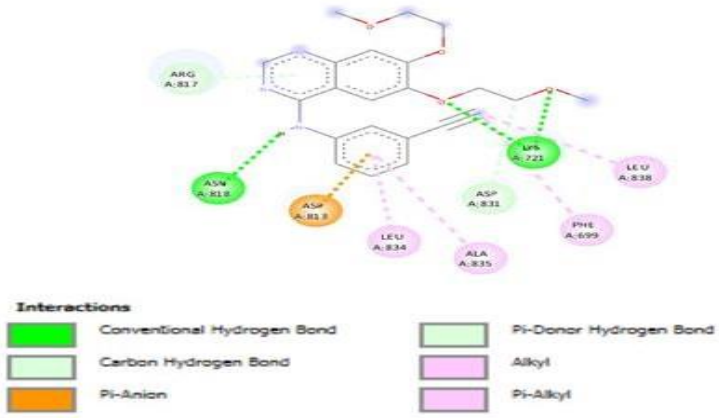


Fig 8: A.A Interactions with ERLOTINIB

Table 8: Interactions of ERLOTINIB with EGFR

Index	Residues	A. A
1	ARG	817
2	ASN	818
3	ASP	813
4	ASP	831
5	ALA	835
6	LEU	834
7	LEU	838
8	LYS	721
9	PHE	699

All the chosen extracted phytochemicals were docked as per the above-described procedure.The binding energies of best binding pose for each were recorded and interaction with the site was studied. The results were compared with the standard drug. The binding energies ofdocked ligand are presented in table 9

Table 9: Binding energies of Phytocompounds

Ligand names	Binding energies	H-bond
Erlotinib	-5.81	Present
Propanamide,2hydroxy	-4.19	Present
1,2 Benzenedimethanol	-4.05	Present
4-Hydroxy-Beta-Ionone	-5.98	Present

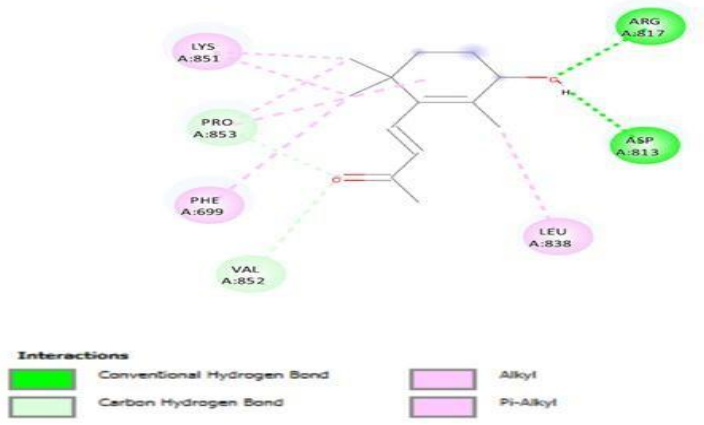


Fig 9: Interactions of 4-hydroxy-beta-ionone with EGFR

Interaction of extracted phytochemicals with EGFR:

Detailed interactions were studied for each ligand: The docking analysis of 4-hydroxy-beta-ionone in the active site of EGFR (PDB ID: 4HJO) protein revealed that it has good binding with the active site, with binding energies as shown in the table with standard reference ERLOTINIB has -5.98 Kcal/mol as its binding energy. The compounds showed similar interactions as that of the standard which mainly involve pi-alkyl interactions with LEU838, PHE699 were observed which can be expected to show the desirable activity.

Docking analysis of extracted phytochemicals with EGFR:
The docking analysis of extracted phytochemicals with the active site of the EGFR revealed that it has a good binding with the active site with binding energy -4 &-6 kcal/mol whereas the reference standard ERLOTINIB has, -5.81kcal/mol as it's binding energy. Interactions ofother extracted phytochemicals with amino acids were noted down in the below table 10.

Table 10: Interaction of phytochemicals with EGFR

Extracted phytochemicals	Interactions
2 propanamide, hydroxy	ASP815, MET957, TRY967, LUE904
1,2benzenedimethanol	LEU838, ALA835, PRO853, PHE699, ASN818, ASP813
4-hydroxy-beta-ionone	LYS851, PRO853, ARG817, ASP813LEU838, VAL852, PHE699

Conclusion

Phytochemicals present in the extract of Neolamarckia Cadamba bark were identified through GC-MS analysis and chemical nature of compounds was noted. Based on these few compounds- propanamide 2 hydroxy;1,2 benzenedimethanol;4- hydroxy-beta-ionone were selected for molecular docking studies on proteins related to MCF, A549, HEPG2 cell lines which areHER2, VEGFR2, EGFR respectively. Binding energies and binding affinity to the target proteins reveals that almost all the compounds have shown remarkable anticancer activity when compared to respective standard substances.

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