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GC-MS analysis of *Croton scabious* Bedd. Extracts & their molecular docking studies for anti-cancer activity against Breast & lung cancer factors

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ABSTRACT

Terpenoids are naturally occurring aromatic or volatile hydrocarbons with isoprene units generally present in the form of essential oil in plants, fungi, and many living organisms. Terpenes demonstrate interesting biological activities such as anti-inflammatory, anti-viral, and anti-cancer activities. The present original research emphasises on the identification of bioactive terpenoids from bark extracts of *Croton scabious* bedd. An endemic plant of Euphorbiaceae family by phytochemical tests and GC-MS analytical methods and some of the important identified terpenoids were docked using Schrodinger Glide software 2019_1 with Human cyclin-dependent kinase 2 complexed with CDK4 inhibitor (PDB ID:1GII) and Human Estrogen receptor α ligand-binding domain in complex with 4-Hydroxy Tamoxifen (PDB ID:3ERT) as breast cancer inhibitors. Crystal structure of the EGFR kinase domain in complex with Gefitinib (PDB ID:2ITY) and Crystal structure of VEGFR2 (juxtamembrane and kinase domains) in complex with sunitinib (PDB ID: 4AGD) was selected to assess lung cancer-inhibiting the potential of these molecules. Fenretinide had revealed significant inhibitory activity on CDK, aromatase, and epidermal growth factor receptors to substantiate anticancer activity against breast & Lung cancers among the identified terpenoids.

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INTRODUCTION

Breast & Lung cancers are second & third most common cancers as per WHO. According to 2018 reports, 2.0-2.1 million cases were reported from which 1.9 million deaths were due to lung cancer. Free radical accumulation in the body due to destructive oxidative stress by toxic pollutants is the main cause of cancers. Excluding the inherited cancers, poor food habits & unhealthy lifestyle are also considered as major concerns for oxidative stress, which leads to developing cancers. Physiological factors like mutation, genetic abnormalities,

and abnormal functioning of some enzymes are major issues for the prognosis of various cancers. In the present study, a special emphasis is made on the selection of enzymes as PDB ids to demonstrate the anticancer effect of phytoconstituents on a particular enzyme complex to perform molecular docking. Cyclin-dependant kinase 2 is an enzyme that plays an important role in the regulation of G_1 to S phase transformation and acts as a checkpoint. Abnormal expression of this enzyme leads to chromosomal instability and hyperproliferation of the cell to develop as cancer cells. CDK inhibitors such as Roscovitine & flavopiridol act by inhibiting non–specific subunits of CDK and proved as best targets to control transcription of carcinogenic cells. A combination of CDK & aromatase inhibitors is generally used as initial endocrine-based therapy in breast cancer. Human cyclin-dependent kinase 2 complexed with the CDK4 inhibitor (1GII) has one unique chain contains 298 residues with resolution 2 A^o expressed from *Spodopteraf rugiperda* of Homo sapiens. The co-crystal ligand 1PU – CDK4 inhibitor 1-(5-Oxo-2,3,5,9b-tetrahydro-1h-pyrrolo[2,1-a]isoindol-9-yl)-3-pyridine-2-yl-urea has binding affinity IC50: 80 - 440nM.

Estrogen receptor α & Estrogen receptor β are nuclear transcription factors of DNA sequences act by binding to mammary glands, uterus ovaries, liver, & adipose tissue as their specific targets. ERβ is predominantly present in the prostate& immune system. Overexpression of ERα causes excessive proliferation in cells of mammary glands and leads to develop breast cancers. ERβ has antagonising effects of ERα by demonstrating growth inhibitory effect on ovarian and all other mammalian cells. ER α antagonists or ER β agonists Fulvestrant is a new drug that shows a promising effect in advanced breast cancer and requires endocrine therapy (Puranik *et al.*, 2019). The human estrogen receptor alpha ligand-binding domain in complex with 4-hydroxytamoxifen (3ERT) has one unique chain contains 261 amino acid residues with resolution 1.9 A^o expressed from *Escherichia coli* of Homo sapiens. The co-crystal ligand 4-Hydroxytamoxifen (OHT) has KI: 0.249nM.

Epidermal growth factor receptor (EGFR) is a protein involved in the regulation of epithelial tissue development and hemostasis. Excessive activation of EGFR leads to amplification of EGFR release and causes mutations in the genome, specifically in lung & breast cancers. The drugs which target biosynthesis pathways to inhibit the EGFR release are used in the treatment of lung & breast cancer. The crystal structure of the EGFR kinase domain in complex with Iressa (Gefitinib) 2ITY has a unique chain contains 327 amino acid residues with resolution 3.42A^oexpressed from *Spodoptera frugiperda* of Homo sapiens. The co-crystal ligand (IRE) has Kd: 53.5nM (Kavitha *et al.*, 2015).

Vascular endothelial growth factor comprises 6subunits as VEGFA – VEGFE and placental growth factors 1&2 complex. VEGF-A is a nuclear factor induces vascular permeability in tumor cells. VEGFR 2 regulates the downstream effects of VEGFA by mediating tyrosine kinase release. Hence, VEGFR 2 are also termed as Tyrosine kinase inhibitors. These can reduce lymphangiogenesis, tumor necrosis factor release during anticancer treatment. Crystal structure of VEGFR2 (juxtamembrane and kinase domains) in complex with sunitinib (SU11248) (N-2-diethylaminoethyl)-5-((Z)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl)-2,4-dimethyl-1H-pyrrole-3- carboxamide) – 4AGD has unique chain contains 353 amino acid residues with resolution 2.81A^oexpressed from *Spodoptera frugiperda* of Homo sapiens. The co-crystal ligand sunitinib has IC50: 5.5 – 18900nM (Prabhu *et al.*, 2014).

Many medicinal plants (Huang *et al.*, 2012; Iqbal *et al.*, 2017) serve as antioxidants and anticancer agents with their lead active constituents such as flavonoids, terpenoids, alkaloids, and vitamins, etc. Sesqui, mono, di & tricyclic terpenoids with isoprene alcohol or ester linkages impart aromatic or volatility to the molecules.

In the present study, acetone, chloroform, and ethyl acetate bark extracts of *Croton scabious* bedd. (Azwanida, 2015; Rao and Salamma, 2013) were prepared by cold maceration method. Phytochemical tests were performed as preliminary identification of diverse secondary metabolites present in all tree bark extracts. GC-MS study was performed for further confirmation of particular secondary metabolites. Identified terpenoids were docked with the above-mentioned enzyme target complexes to correlate anticancer mechanisms against breast & lung cancers.

MATERIALS AND METHODS

Croton scabious Bedd bark was crushed to give a moderately coarse powder. Every 250 grams of powder is placed in three different round bottom flasks RBFs labeled as M_1, M_2, and M_3, then 750ml of selected solvents such as Acetone, Chloroform, and Ethyl Acetate was added to M1, M2, and M_3 respectively. RBFs were kept in cool & dark place and allowed to stand for seven days with occasional shaking. Solvents were recovered by separating the marc using a rota evaporator. Finally, solvents were

concentrated to obtain extracts (Azwanida, 2015).

Phytochemical tests (Sheel *et al.*, 2014; Tiwari *et al.*, 2011) were performed on Acetone (M_1), Chloroform (M_2), and Ethyl acetate (M_3) bark extracts to identify various secondary metabolites present in *Croton scabious* Bedd. The results obtained were tabulated in the results section.

GC-MS analysis

The Gas Chromatography-Mass Spectroscopy (GC–MS) analysis of the components were carried out as per the method developed by sangggil Choe et al., with slight modifications equipped with mass-selective detector (MSD) (HP6890 GC and HP7673) autosampler, operated at 65eV using acquisition scan mode with HP-5MS (GC capillary column, 0.25 mm$\times$0.25 μm$\times$30 m) at 100°C oven temperature held initially for 1 min and then increased to 280°C by 20°C/min and held for 10 min. 250°C was the injector temperature, and Helium was used as the carrier gas at a constant column flow rate of 1.5 ml/min. By split, less mode technique 2μl aliquot of the sample extract was injected. From 10 chromatograms, target components were selected, and Comp Extractor software was used for the registration of chromatograms. The identified bioactive components were schematically represented in results section (Kanthal *et al.*, 2014).

Virtual Screening (Molecular Docking)

Ligand Preparation

The extracted chemical compounds structures were imported into maestro Schrodinger software by downloading in SDF (Structure Data File) format from PubChem online database. By selecting all ligands in the side view, then they were subjected to Ligprep in the task menu, and required parameters Ionization (Neutralize), chirality, computation, etc. were selected and program run by giving job name, in working directory file previously created (Mctigue *et al.*, 2012; Muchtaridi *et al.*, 2018).

Macromolecule (Protein) preparation

The protein structure imported by giving the code 1GII in protein preparation wizard (it uses protein data bank (PDB) online database to import the protein). Then the protein is preprocessed by assigning bond orders, adding hydrogens, create disulfide bonds, etc. Next, in the review and Modify tab, it showed two water molecules with residue numbers 661 and 664. Among these two water molecules residue, 664 has interactions with ligand and proteins, residue 661 does not show any interactions, and it was deleted. The co-crystal ligand Het IPU (501) was kept as such. Further, refine the tab, the first protein was optimized, then it is minimized. The two-dimensional Ramachandran plot shows the allowed and disfavored values of ψ and φ. The Ramachandran plot is a plot of the torsional angles - phi (φ)and psi (ψ) - of the residues (amino acids) contained in a peptide represented in figure 1. Similarly, the protein 3ERT, 4AGD, and 2ITY structures were also imported and subjected to minimization. The Ramachandran plot of these proteins was represented in Figure 1.

In maestro task, selected receptor grid generation–generated grid suitable for peptide docking by selecting any one atom of the ligand molecule on screen; it shows grid box with X, Y, and Z coordinates 6.43, 9.76 and 26.06 respectively. Then the remaining options - site, constraints, and rotatable groups were enabled, and the grid box was executed by clicking run.Similarly, the grid box generated for 3ERT protein with X, Y and Z coordinates 31.91, -1.79 and 25.15; 4AGD protein with X, Y and Z coordinates 51.6, -1.62 and -15.4; 2ITY protein with X, Y and Z coordinates -49.76, -1.26 and -20.73 (Mctigue *et al.*, 2012; Muchtaridi *et al.*, 2018).

Ligand docking

In the maestro task, Ligand docking (virtual screening) was selected. In the ligand docking tab, the Glide grid and ligand outmaegz zip files were loaded from the working directory. Then, in settings, write XP descriptor information is selected and clicked on the run. The virtual screening results were indexed as per maximum binding energy to a minimum with amino acid residues of 1GII, 3ERT, 4AGD, and 2ITY (Mctigue *et al.*, 2012; Muchtaridi *et al.*, 2018).

RESULTS AND DISCUSSION

Alkaloids, Terpenoids, Flavonoids, Cardiac glycosides, Steroids, and Carbohydrates were identified as major constituents in Chloroform and ethyl acetate extracts, whereas alkaloids, carbohydrates, flavonoids, and tannins were found to be absent in acetone extract Table 1.

Azulene, 1,4dimethyl7(1 methyl ethyl), Fenretinide, Retinal, Cedarn 8S, 13-diol, Ylangene, alfa. Copaene, Calamenene, Geranyl- à –terpinene were identified as chief terpenoids in all the three bark extracts by comparing the retention time and peak % areas of standard GC-MS spectra's as shown in Table 2 and Figure 2.

The extracted compounds were docked against different lung and breast cancer protein sequences 1GII, 3ERT, 4AGD, and 2ITY to know the interactions and docking score by Glide Schrodinger docking software and the results were reported inTable 3.

Table 1: Qualitative analysis of phytochemical constituents

Chemical tests	Croton scabious Bedd. Bark extract		
	Acetone (M1)	Chloroform (M2)	Ethyl acetate (M3)
Mayer's test	+	++	+++
Dragendorff's test	-	++	+++
Wagner's test	-	++	+++
Hager's test	+	++	+++
Terpenoids test	+++	+++	+++
Tannic acid test	+++	+	+++
Legal's test	+	++	+++
Froth test	-	+++	+++
Hemolysis test	-	++	++
Gelatin test	+	+	+
Ferric chloride test	+++	+++	+++
Alkaline reagent test	+++	+++	+++
Shinoda test	-	++	+++
Zinc-Hydrochloride reduction test	+	++	+++
Alkaline reagent test	+	+++	+++
Millons test	++	-	++
Ninhydrin test	+	-	+
Molisch's test	-	++	++
Benedicts test	-	++	++
Fehlings test	-	++	++
Phosphates test	++	++	++
Sulphates test	++	++	++

+ + +: High presence of phytochemical constituents
+ +: Moderate presence of phytochemical constituents
+ : Lowpresence of phytochemical constituents
- : Absence of phytochemical constituents

Table 2: GC-MS data of Phytocompounds

S. No	Compound Name	Molecular formula	RT	Area %
1	Azulene	$C_{15}H_{18}$	12.92	1.56
2	Fenretinide	$C_{26}H_{33}NO_2$	24.16	2.52
3	Limonen-6-ol, pivalate	$C_{40}H_{52}O_4$	22.47	3.44
4	Retinal	$C_{20}H_{28}O$	23.95	2.83
5	Cedarn 8S, 13-diol	$C_{15}H_{26}O_2$	24.36	4.73
6	Ylangene	$C_{15}H_{24}$	9.26	1.21
7	alfa.Copaene	$C_{15}H_{24}$	9.27	1.46
8	Trans Calamenene	$C_{15}H_{22}$	9.90	1.37
9	Geranyl- α -terpinene	$C_{20}H_{32}$	21.35	2.18

Table 3: Docking score for the selected terpenoids by Glide software

Name of the Compound	Docking Score			
	1GII	3ERT	4AGD	2ITY
Fenretinide	-6.435	-8.333	-5.444	-2.397
Retinal	-5.792	-7.205	-4.624	-3.537
Trans calamine	-5.473	-8.013	-3.514	-3.939
Azulene	-5.001	-5.246	-6.513	-3.044
Alfa ylangene	-4.454	-8.165	-2.325	-3.157
Cedran-8,13-diol	-4.435	-7.573	—	-3.129
Geranyl alpha terpene	-4.345	-7.577	-4.904	-4.34
Limonene-6-ol, Pivalate	-4.008	-6.69	-5.808	-3.991
Alfa copaene	-3.609	-8.133	-1.997	-2.84
Ingol-12-acetate	—	—	-2.276	-4.705
CDK4 Inhibitor	-7.717	—	—	—
4-Hydroxy Tamoxifen	—	-11.891	—	—
Sunitinib	—	—	-11.93	—
Gefitinib	—	—	—	-5.776

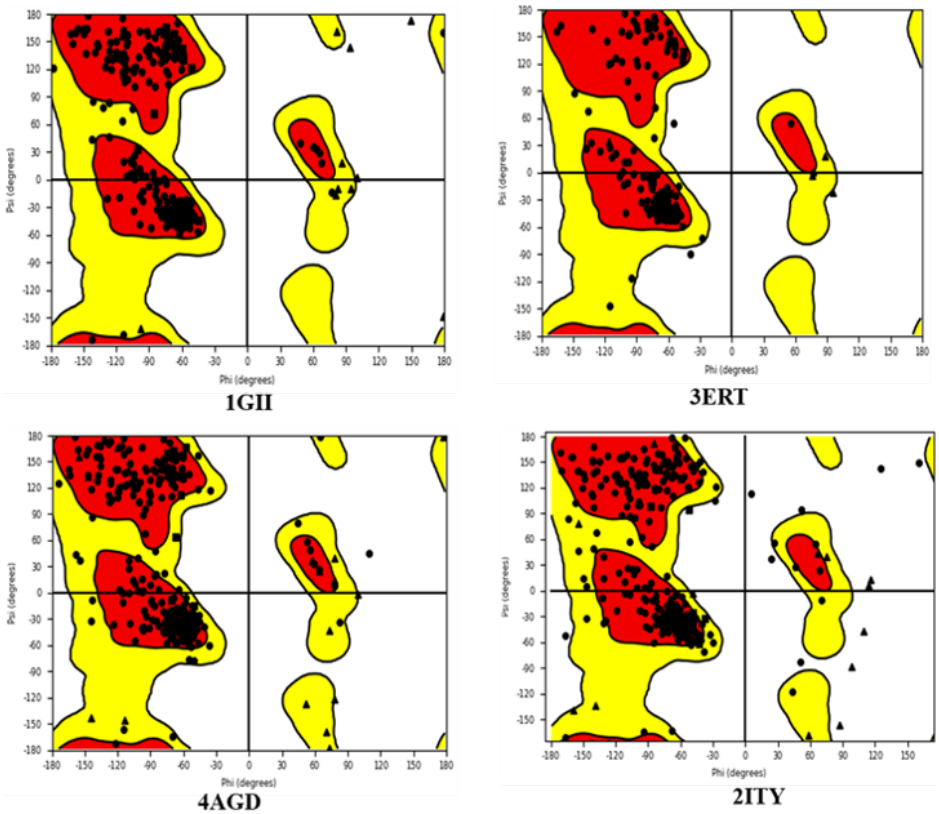

Figure 1: Ramachandran two-dimensional plot shows the allowed and disfavored values of ψ and φ of the amino acid residues in a proteins 1GII, 3ERT, 4AGD and 2ITY Receptor grid generation

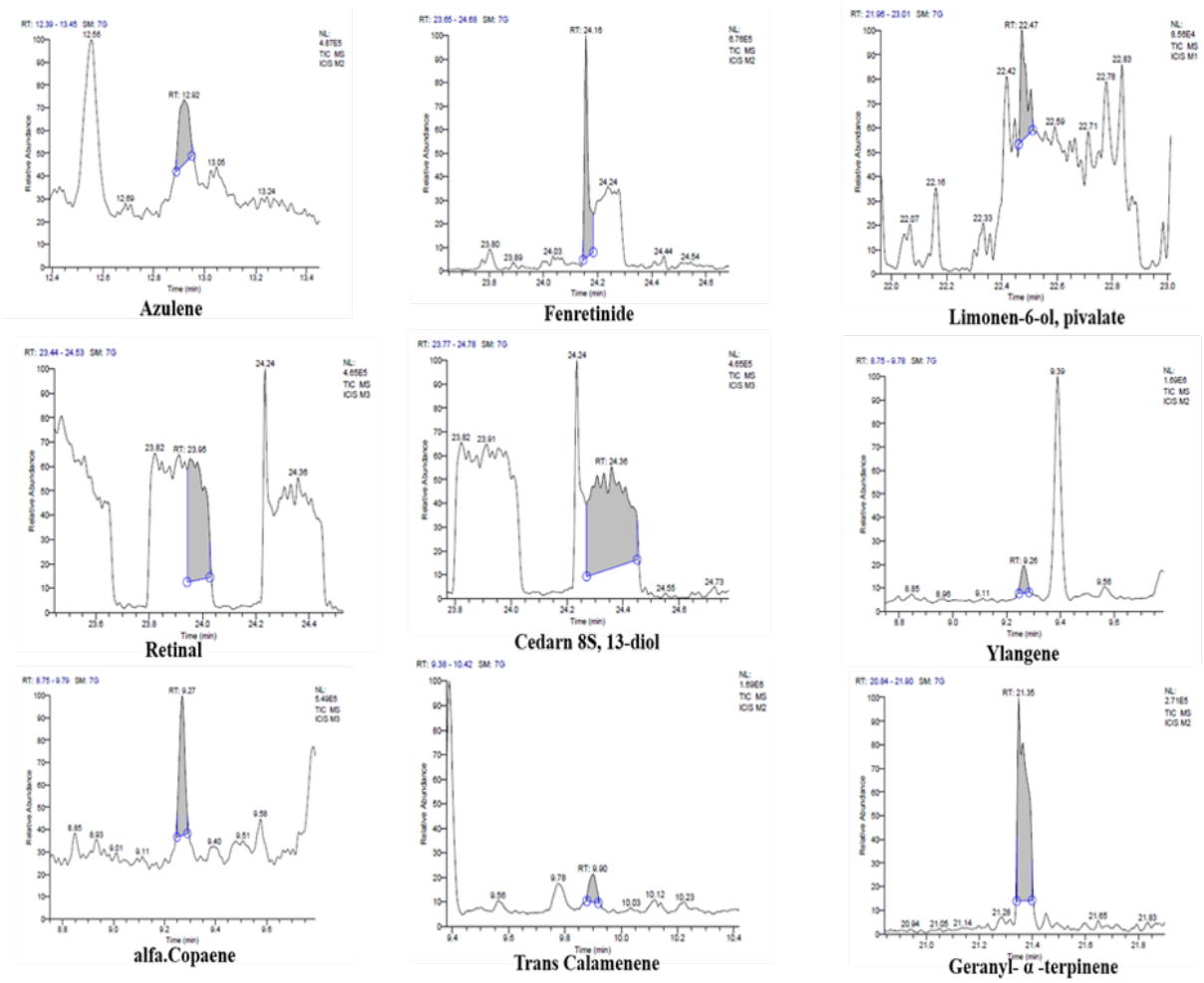

Figure 2: GC-MS Chromatograms of selected terpenoids

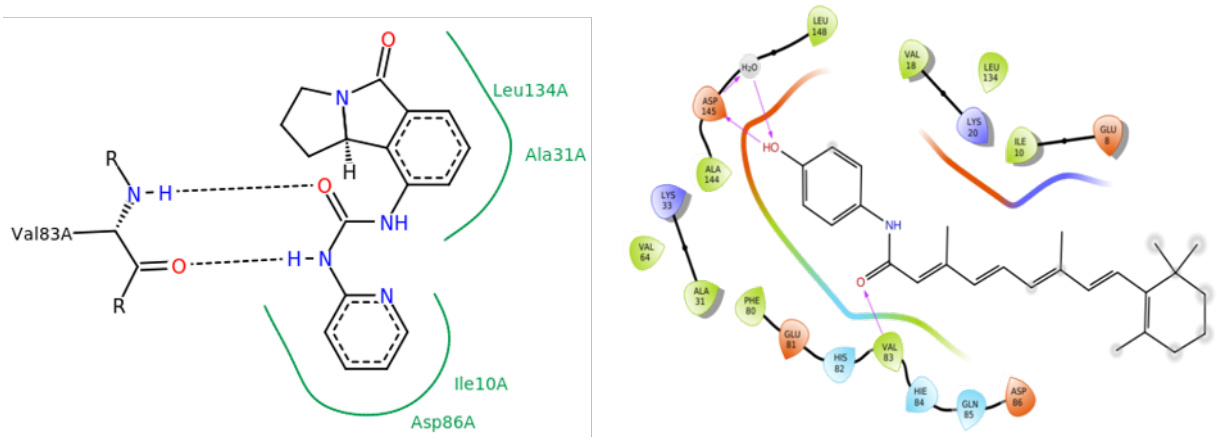

Figure 3: 2D interaction diagrams of CDK4Inhibitor and Fenretinide with Human Cyclin-Dependent Kinase 2(Chain A;PDB gene code: 1GII)

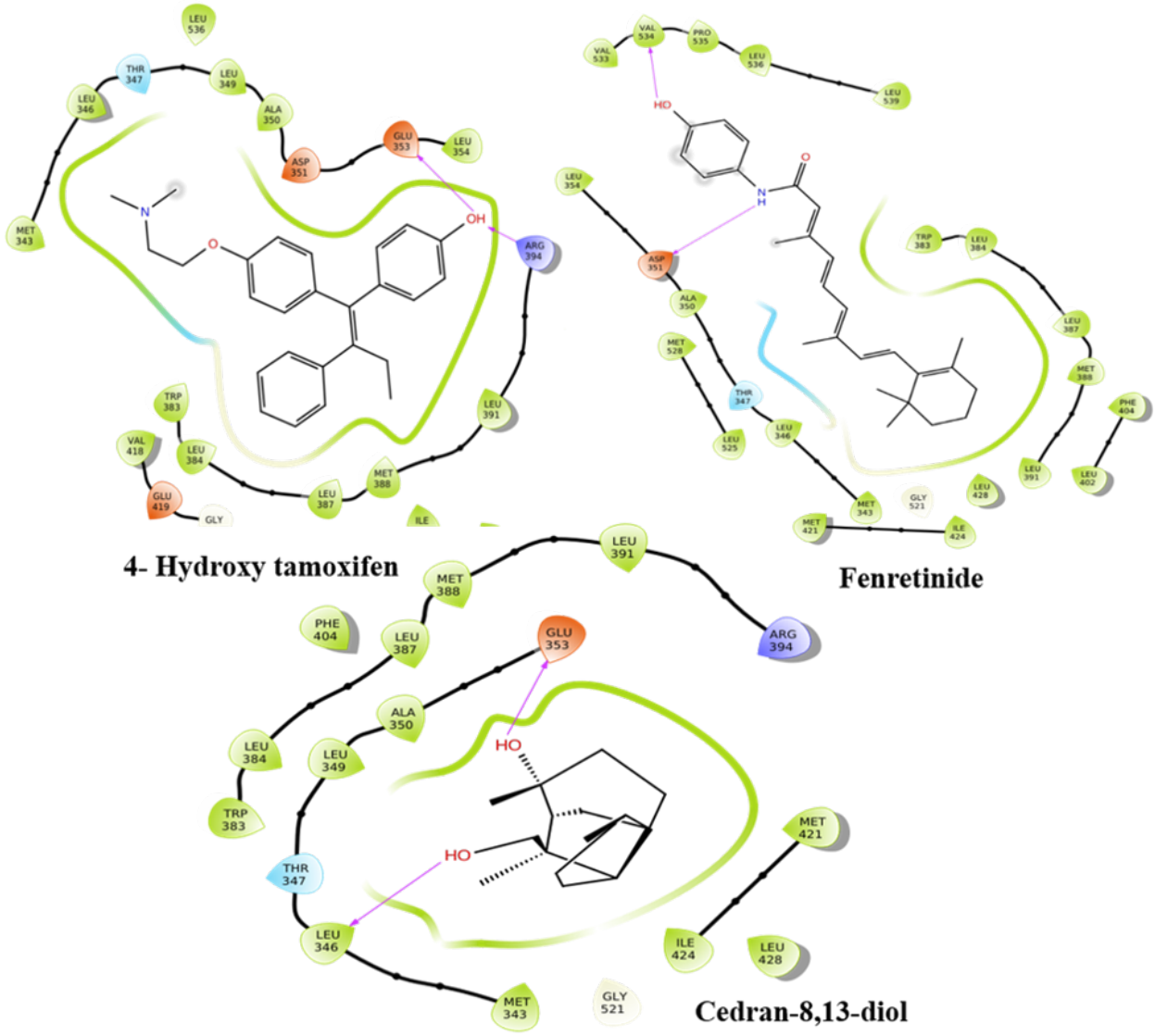

Figure 4: 2D interaction diagrams of 4-Hydroxy tamoxifen, Fenretinide, and Cedran-8,13-diol with Human estrogen receptor alpha ligand-binding domain (Chain A; PDB gene code: 3ERT)

From the docking result, it revealed that the compounds were showed good binding interaction with the Human estrogen receptor alpha ligand-binding domain in complex with 4-hydroxytamoxifen (3ERT). The CDK4 inhibitor, which is co-crystal ligand in the 1GII **(**Human Cyclin-Dependent Kinase 2) protein showed two H-bonding interactions (Docking score -7.717) with amino acid residue Val 83, similarly among the docked compounds, Fenretinide showed very good H-bonding interactions (Docking score -6.435) with amino acid residues Val 83 and Asp 145, represented in Figure 3.

4-Hydroxy tamoxifen, which is co-crystal ligand in the 3ERT **(**Human estrogen receptor alpha ligand-binding domain) protein, showed two H-bonding interactions (Docking score -11.891) with amino acid residue Arg394 and Glu353. Among the docked compounds, Fenretinide showed good H-bonding interactions (Docking score -8.333) with amino acid residues Asp351 & Val 534, and the compound Cedran-8,13-diol represented H-bonding interactions (Docking score -7.573) with amino acid residues Leu346 & Glu353 as shown in Figure 4.

Sunitinib, which is co-crystal ligand in the 4AGD (Crystal structure of VEGFR2 juxtamembrane and kinase domains) protein, showed four H-bonding interactions (Docking score -11.93) with amino acid residues Glu917, Cys919 and Leu840, Hydrophobic interaction with Phe1047 amino acid. From the docking data, the compound Azulene occupied the active site of the protein with dock score -6.513, but it does not show any H-bonding interactions with amino acid residues. But the compound Limonene-6-of Pivalate showed H-bonding interaction (Dock-

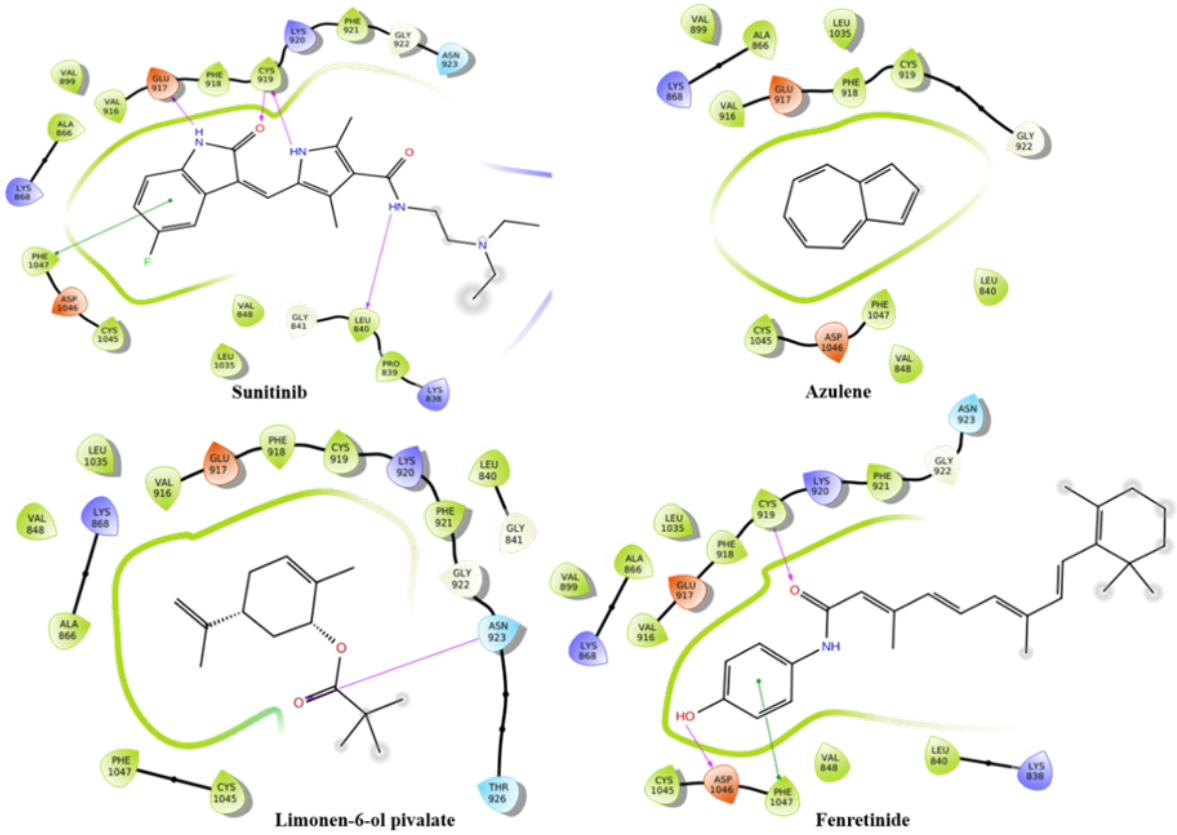

Figure 5: 2D interaction diagrams of Sunitinib Azulene, Limonene-6-olpivalate, and Fenretinide with Crystal structure of VEGFR2 (Chain A; PDB genecode: 4AGD)

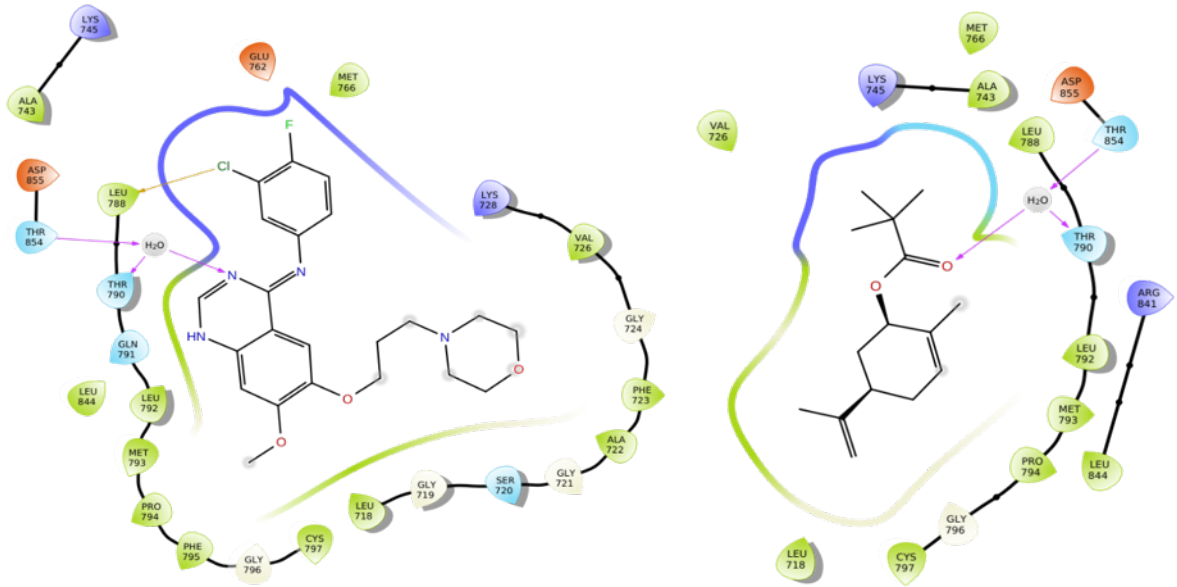

Figure 6: 2D interaction diagrams of Gefitinib and Limonene-6-olpivalatewith Crystal structure of EGFR kinase domain (Chain A; PDB gene code: 2ITY)

ing score -5.808) with amino acid residue Asn923 and also Fenretinide represented good H-bonding interactions (Docking score -5.444) with amino acid residues Asp1045 & Cys919 and Hydrophobic interaction with Phe1047 as shown in Figure 5.

Gefitinib, which is co-crystal ligand in the 2ITY (Crystal structure of EGFR kinase domain) protein, showed H-bonding interactions (Docking score -5.776) with water residue 2019, (water residue makes H-bond interaction with ligand and amino residues Thr790 and Thr854) and Halogen bond with amino acid residue Leu788. The similar interactions with water residue 2019 are observed in compound Limonene-6-of pivalate with docking score -3.991, as shown in Figure 6.

CONCLUSION

Fenretinide has shown considerable docking score to exhibit aromatase & estrogen receptor α inhibition; hence, they demonstrated anti-cancer activity against breast cancer when compared with CDK4 inhibitor & Tamoxifen. Azulene has shown good docking score to exhibit EGFR inhibiting potential so as to reveal anti lung cancer activity when compared with Sunitinib. Ingol-12-acetate exhibited significant docking scores against VEGFR 2 inhibition, which may explicate anti-cancer activity against lung cancer cells. But no docking was observed against breast cancer-inhibiting enzyme complexes. Cedran-8, 13-diol was not able to dock with 4AGD hence revealed as a poor inhibitor of EGFR. Based on the above remarks on molecular docking, this research study can be further continued for *the in-vitro* evaluation of the anticancer activity of *Croton Scabiosus* Bedd.

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