ORIGINAL ARTICLE

Molecular docking analysis between anti-apoptosis EGFR and four coumarins, and four carbazole alkaloids: in silico study

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ABSTRACT

Introduction: The anti-apoptotic protein EGFR is typically overexpressed in the majority of head and neck squamous cell carcinomas (HNSCC) and has been targeted for genetic therapy. The *Clausena excavata* plant is an evergreen shrub that has been widely used for various disease therapies, including cancer. Coumarin and carbazole alkaloids are the plant's primary active ingredients. This study aims to determine the molecular interaction between EGFR and several coumarins (clauslactone E, dentatin, nordentatin, clausenidin) and carbazole alkaloids (7- hydroxyheptaphylline, clausine E, 2,7 - dimethoxy - 9H - carbazole - 3- carbaldehyde,and 2,7 – dimethoxy - 9H - carbazole – 3-carboxylic acid). **Methods**: This research was carried out *in silico* using the molecular docking method. Molecular docking analysis was performed using AutoDock Vina, AutoDockTools 1.5.6., Pymol, and Discovery Studio Biovia 2021. The three-dimensional structure of the EGFR protein was retrieved from the RCSB Protein Data Bank. Ligands were obtained from the PubChem Compound Database. The comparison ligand was doxorubicin. Molecular docking results were analyzed based on binding affinity, amino acid interactions, visualization of docking results, and Lipinski's rule of five. **Results**: All of the investigated ligands with the EGFR receptor had strong binding affinity (-6.8 and -8.3 kcal/mol), almost the same as the comparison ligand (-8.2 kcal/mol). Each interaction also produced a different number of amino acid residues. **Conclusion**: These four coumarin compounds and four carbazole alkaloid compounds are considered potential EGFR inhibitors and anticancer candidates.

KEYWORDS
Molecular docking, anticancer, oncogene protein, natural compunds

INTRODUCTION

The process of "programmed cell death," or apoptosis, is a critical regulator of cell proliferation and tissue development.[1] Disturbances in the control of apoptosis may result in resistance to chemotherapeutic agents.[2] The apoptotic pathway is a promising target for creating new anticancer therapies because it is not specific to the type of cancer. Compounds derived from plants are compounds that are very promising for triggering apoptosis and are not toxic to healthy cells.[3]

The human epidermal growth factor receptor (EGFR) is an oncogenic gene thought to contribute to the development of neck and head cancer (HNSCC). EGFR impacts cell motility, metastasis, adhesion, angiogenesis, gene expression, proliferation, and apoptosis inhibition.[4] EGFR dysregulation, an early event of neck

and head cancer, is associated with disease aggressiveness, resistance to chemotherapy, and poor survival.[5]

A poor prognosis is linked to EGFR overexpression or the presence of mutations in up to 80–90% of HNSCCs. These alterations contribute to carcinogenesis and have a direct effect on overall survival and progression-free rates.[6-8] EGFR mutations and overexpression are associated with a variety of tumors, including HNSCC, making EGFR one of the main targets for developing promising molecular interventions.[8,9]

Clausena excavata is an uncultivated shrub belonging to the Rutaceae family. It mostly grows in Southeast Asian countries.[10,11] *C. excavata* has been empirically used to treat illnesses of the respiratory tract, throat, wound healing, stomach ailments, and others.[12] According to a phytochemical study, C. *excavata* has a significant quantity of coumarins, carbazole alkaloids, and small amounts of flavonoids, limonoids, and triterpenoids. Coumarin and carbazole alkaloids are the main constituents of the plant.[11] These compounds were found to have various pharmacological effects, such as antibacterial, antifungal, analgesic, anti-inflammatory, antiviral, antioxidant, immune-modulator, and anticancer activity.[10,13] Among the coumarins, nordentatin and dentatin showed strong cytotoxicity against some cancer cell lines.[14,15]

For developing new drugs, computational protein-ligand docking *in silico* has become a crucial technique. Through molecular docking, a complex between a receptor and a ligand structure and binding affinity is predicted, represented as a protein-ligand binding force in kilocalories per mole (kcal/mol).[16,17] Computational docking research generates a virtual model of protein-ligand interactions at the atomic level, which is a straightforward and logical approach to drug development. The *in silico* approach offers a significant advantage before doing *in vivo* lab studies, as it can reduce the time and expense associated with the drug development process.[18] So far, there has been little in-depth research on the use of in silico protein-ligand docking to predict protein interactions with potential compounds such as coumarins and carbazole alkaloids.

AutoDock Vina was used in this study because it is faster, more accurate, and more effective for most systems.[17-19] This study aims to determine the molecular interaction between EGFR and several coumarins (clauslactone E, dentatin, nordentatin, clausenidin) and carbazole alkaloids (7 - hydroxyheptaphylline, clausine E, 2,7 – dimethoxy - 9H - carbazole - 3 - carbaldehyde, and 2,7 – dimethoxy - 9H - carbazole – 3 - carboxylic acid).

METHODS

This research method was in silico *simulation* with molecular docking of four coumarin compounds (clauslactone E, dentatin, nordentatin, and clausenidin) and four carbazole alkaloid compounds (7 - hydroxyheptaphylline, clausine E, 2,7 - dimethoxy - 9H - carbazole - 3 - carbaldehyde, and 2,7 - dimethoxy - 9H - carbazole - 3 - carboxylic acid) with the anti-apoptotic protein EGFR. In this study, doxorubicin was the comparison ligand. Ligands were obtained from the PubChem Compound Database in Spatial Data File (SDF) format. All investigating ligands used in this study met Lipinski's criteria (the Lipinski's rule of five), as seen in Table 1. A compound that meets Lipinski's criteria means that the compound has good oral bioavailability. The five criteria of Lipinski's rule state that poor absorption or permeation of a compound is typically observed if: (1) it has more than five donors of hydrogen bonds; (2) a molecular weight of more than 500; (3) C log P (calculated octanol/water partition coefficient) greater than five; (4) more than ten H-bond acceptors are present (the amount of nitrogen and oxygen is more than 10).[20] From the RCSB Protein Data Bank, the EGFR protein's three-dimensional structure was retrieved, PDB code: 5FED. The main docking application used in this experiment was AutoDock Vina. The EGFR PDBQT (Autodock structure) files were prepared and grid box sizes were determined

using AutoDock Tools version 1.5.6 by the Scripps Research Institute in La Jolla, California, USA.

Table 1. The ligand's physicochemical characteristics based on Lipinski's rule of five

Ligands	Pubchem id	Molecular formula	Molecular weight (<500g/mol)	Hydrogen binding acceptors (<10)	Hydrogen binding donors (<5)	Log P (<5)	Rotatable bond count	Meet Lipinski's RO5 Criteria
Coumarin Compounds								
1.Clauslactone E 2.Dentatin 3.Nordentatin	10450031	$C_{19}H_{18}O_6$	342.3	6	1	3.5	5	Yes
4.Clausenidin	342801	$C_{20}H_{22}O_4$	326.4	4	0	4.7	3	Yes
	5320206	$C_{19}H_{20}O_4$	312.4	4	1	4.4	2	Yes
	5315947	$C_{19}H_{20}O_5$	328.4	5	1	4.1	2	Yes
Carbazole alkaloid compounds								
1.7-Hydroxyheptaphylline	15767846	$C_{18}H_{17}NO_3$	295.3	3	3	4.6	3	Yes
2.Clausine E 3.2,7-dimethoxy-9H-carbazole-3-carbaldehyde	5315951	$C_{14}H_{11}NO3$	241.24	3	2	2.9	2	Yes
	5317755	$C_{15}H_{13}NO_3$	255.27	3	1	2.8	3	Yes
4.2,7-Dimethoxy-9H-carbazole-3-carboxylic acid	504070	$C_{15}H_{13}NO_4$	271.27	4	2	2.9	3	Yes
Doxorubicin	31703	$C_{27}H_{29}NO_{11}$	543.5	12	6	1.3	5	No

Using Discovery Studio Biovia 2021, the chemical structure of each ligand was converted from SDF file format to PDB file format. AutoDockTools 1.5.6. (ADT) was used to change the structure of "ligan.pdb" to "ligan.pdbqt", making it eligible for AutoDock Vina usage. The EGFR protein was downloaded from the PDB database with the code PDB: 5FED. To validate docking and conduct further ligand-based virtual screening, the ligand that was present in the protein was separated and stored as pdb files. For structure-based virtual screening and molecular docking methods, the EGFR protein structure was prepared by having the water molecules' atomic coordinates removed, polar hydrogen added, Gasteiger charges for protein structures calculated, and protein structures converted from PDB file format to PDBQT format.[21]

For docking validation, the native ligands on the target protein should be redocked. Utilizing Biovia Discovery Studio 2021, the native ligand was dissociated from the protein. In this study, the native ligand (~{N}-[7-methyl-1[(3~{R})-1-propanoylazepan-3-yl] benzimidazole -2-yl] -3 (trifluoromethyl) benzamide) was docked to the EGFR protein (PDB code:5FED). Using the scoring system included in AutoDock Vina, the expected binding affinity (kcal/mol), which gauges how tightly a ligand binds to the receptor, is computed. A stronger binding is indicated by a larger negative binding affinity. The approach is considered valid if the RMSD value generated is ≤2A when the test compound and target protein can be docked in the same grid box region.[22,23]

The AutoDock Vina program was used for molecular docking. The receptor was docked with each ligand, with a binding site obtained through the grid box (specific center and size). In this work, the binding site was obtained by re-docking the ligands contained in the protein. Each ligand had a flexible state and interacted with stiff macromolecules. AutoDock Vina was used in the docking simulation. To start AutoDock Vina, Notepad was used to prepare the setup file. The conformation of docking was established by selecting the position with the largest negative binding affinity (the highest affinity). The docking results were shown using Discovery Studio Biovia 2021 and PyMol (DeLano Scientific LLC, USA).

RESULTS

To validate the results, the native ligand ((~{N} - [7 – methyl - 1 - [(3~{R}) propanoylazepan -3 - yl] benzimidazol - 2 - yl] - 3 - (trifluoromethyl)benzamide) redocking to the EGFR protein (PDB code: 5FED) has been carried out. The result of redocking was a grid box with center x = -2.260, y = 51.852, z= -20.19, and

size x=10, y=8 and z=12. This redocking produced a binding affinity of -8.5 kcal/mol. The results of the validation are presented in Figure 1.

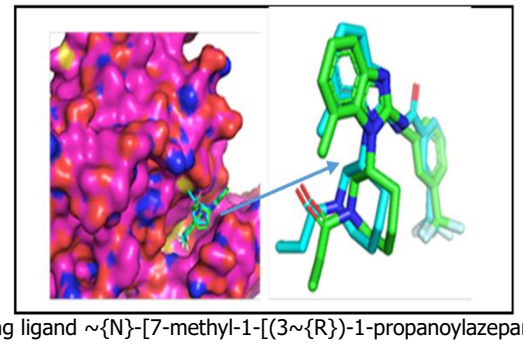

Figure 1. Redocking ligand ~{N}-[7-methyl-1-[(3~{R})-1-propanoylazepan-3-yl]benzimidazol-2-yl]-3-(trifluoromethyl)benzamide. The redocking crystal structure in the best position is shown in green, the conformational ligand crystal structure is blue.

The molecular docking simulation identified the amino acid residues and binding affinities for the interactions of the four coumarin and four carbazole alkaloid ligands with the EGFR receptor. The values of binding affinity and amino acid residues implicated in hydrogen binding, hydrophobicity, and electrostatics are shown in Table 2 and Figure 2.

Ligand-specific interactions with amino acid residues close to the receptor site are characterized by the binding affinity of the ligand to the receptor20. All of the proposed compounds had strong binding affinities ranging from -6.8 to -8.3 kcal/mol. Clausenidin demonstrated the highest binding affinity for the EGFR binding site, measuring -8.3 kcal/mol. The binding affinity of doxorubicin as a comparison ligand was -8.2 kcal/mol.

The amino acid residues involved in the interaction of the ligand with the EGFR receptor, according to hydrogen bonds, hydrophobic interactions, and electrostatic interactions, are shown in Table 2. Table 2 shows that the majority of the amino acid residues engaged in the interaction of both coumarin and carbazole alkaloid ligands with the EGFR receptor participate in hydrophobic interactions. Amino acid residues are less susceptible to hydrogen bonding. Moreover, the nordentatin compound shows no involvement of amino acids in hydrogen bonds or electrostatic interactions. In all interactions of the ligands with the EGFR receptor, there are no amino acid residues generated in the electrostatic interactions.

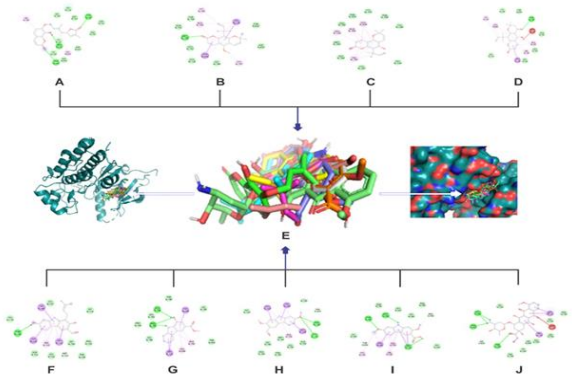

Figure 2. Best conformation model and binding interactions of coumarin compounds (clauslactone E (A), dentatin (B), nordentatin (C), clausenidin (D)), carbazole alkaloid compounds (7-hydroxyheptaphylline (F), clausine E (G), 2,7-dimethoxy-9H-carbazole-3-carbaldehyde (H) and 2,7-dimethoxy-9H-carbazole-3-carboxylic acid (I)) and doxorubicin (J) on EGFR protein. Superimposition of all ligands at the best binding site position (E).

Table 2. The importance of binding affinity and the residues of amino acids that are involved in the interaction between the four Coumarin and four Carbazole alkaloid compounds' ligands and the EGFR receptor at their optimal binding site positions. The binding affinity of the investigated ligands show particular interactions with the residues of amino acids around the receptor site.

Coumarin ligands	Binding Affinity (kcal/mol)	Distance (Å) and amino acids involved		
		hydrogen binding interaction	hydrophobic interaction	electrostatic interaction
Clauslactone E	-6.8	Arg841 (2.92) Thr790 (3.06) Thr854 (3.13)	Leu844 (4.92) Val726 (5.11)	-
Dentatin	-7.9	Met793 (2.8)	Val726 (3.92), Leu844 (3.95), Val726 (4.16), Ala743 (3.64), Lys745 (4.08), Cys797 (3.92), Val726 (4.89), Ala743 (4.54), Leu844 (5.07)	-
Nordentatin	-7.9	-	Val726 (4.65), Leu718 (5.02), Val726 (5.01), Ala743 (4.69), Leu844 (5.10), Val726 (4.16), Ala743 (5.49), Leu844 (4.83)	-
Clausenidin	-8.3	Thr790 (2.81)	Leu718 (3.66), Leu844 (5.13), Cys797 (5.08), Val726 (5.44), Ala743 (5.37), Val726 (4.58), Ala743 (4.4), Leu844 (4.64)	-
Carbazole alkaloid ligands				
7-Hydroxyheptaphylline	-7.3	Thr790 (2.25) Met793 (3.44)	Leu718 (3.57), Val726 (3.71), Leu844 (3.54), Val726 (5.18), Leu718 (5.32), Ala743 (4.45), Leu844 (5.14), Val726 (5.07), Ala743 (3.61)	-
Clausine E	-7.3	Gln791 (2.68) Met793 (2.5) Met793	Leu718 (3.73), Leu718 (3.7), Val726 (3.97), Ala743 (4.17), Leu844 (4.74), Val726 (5.28), Ala743 (4.95)	-
2,7-Dimethoxy-9H-carbazole-3-carbaldehyde	-7.0	Cys775 (3.78) Thr790 (3.1) Thr854 (3.6)	Leu718 (3.81), Val726 (3.69), Leu844 (3.61), Cys775 (4.65), Val726 (5.03), Leu718 (5.3), Ala743 (4.72), Leu844 (5.2), Val726 (5.0), Ala743 (3.63)	-
2,7-Dimethoxy-9H-carbazole-3-carboxylic acid	-7.2	Thr790 (3.13) Pro794 (3.65) Thr854 (3.74)	Leu718 (3.62), Leu718 (3.81), Leu844 (3.87), Cys775 (4.73), Leu844 (5.16), Val726 (5.34), Ala743 (4.71), Leu844 (5.41), Val726 (4.67), Ala743 (3.9)	-
Comparison ligand				
Doxorubicin	-8.2	Arg841 (2.36), Asn842 (2.1), Arg841 (2.6), Thr854 (2.84), Asp855 (3.49)	Leu718 (3.80), Leu718 (3.73), Val726 (3.90), Leu718 (5.01)	-

DISCUSSION

Molecular docking is a computer process performed on structure-based rational drug design to predict the non-covalent bonding of small molecules (ligands) and macromolecules (receptors) and also to gauge how strongly the protein-ligand interaction—typically involving one receptor and one ligand—interacts.[24] Molecular docking is now used as a quick and affordable computational method

to enhance drug development.[25] In order to create structure-based medications as well as understand biomolecular interactions and their processes, docking is frequently utilized.[17]

Figure 2 shows the docking results between four coumarin and four carbazole alkaloid compounds at the best binding site positions. The binding affinity of the coumarin compounds was found to be between -6.8 and -8.3 kcal/mol. The bond between the clausenidin compound and EGFR exhibited the highest binding affinity, measuring -8.3 kcal/mol. Meanwhile, the binding affinity of carbazole alkaloids with EGFR receptors ranged from -7.0 to -7.3 kcal/mol. We demonstrated for the first time that compounds showed optimum binding affinities with EGFR.

The comparison ligand was doxorubicin, with 8.2 kcal/mol binding affinity, not much different from the investigating ligand (table 2). The binding affinity resulting from the above various ligands with the EGFR receptor differed in each interaction. The results showed a strong binding affinity (ΔG) for all ligand interactions of coumarin compounds and carbazole alkaloid compounds with the EGFR receptor.

Types of molecular interactions with essential amino acid residues, such as hydrogen bonds, hydrophobic, and electrostatic interactions, are indicative of ligand docking in favorable conformations, even though binding affinity values are informative of ligand docking in the active pocket of a protein.[15] Our findings reveal that distinct amino acid residues mediate hydrogen bond, hydrophobic, and electrostatic interactions in every ligand-protein interaction. In particular, doxorubicin, as a comparison ligand, forms hydrophobic interactions with only four residues and two types of amino acids. However, these residues are the same as the residues involved in binding to all investigated ligands for val726, but for Leu718 they are absent in binding the dentatin-EGFR and clauslactone-EGFR. (table 2)

Almost all investigated ligands produced more amino acid residues in hydrophobic interactions than in hydrogen bonds, with the exception of the clauslactone E-EGFR bond and the comparison ligand doxorubicin-EGFR bond, which produced more amino acid residues in hydrogen bonds. No amino acid residues were generated upon hydrogen bonding between nordentatin and EGFR. For all ligands, most of the amino acid residues produced occurred in hydrophobic interactions, both for coumarin group compounds and carbazole alkaloid group compounds. Fewer hydrogen bonds produce amino acid residues. For all ligands, there were no electrostatic interactions that resulted in amino acid residues. (table 2)

The formation of stable protein complexes is attributed to strong subunit interactions. Hydrophobic forces were previously thought to be a key factor in forming stable protein complexes. Stable complex formation requires the involvement of additional forces like hydrogen bonds, salt bridges, and Van der Waals interactions.[26] A type of property of nonpolar molecules (or hydrophobic moieties of amphiphiles) that can cause these molecules to assemble to form anhydrous domains in an aqueous solution is called hydrophobic interaction, also referred to as the hydrophobic effect. The hydrophobic effect essentially stems from the entropy effect that occurs when nonpolar solutes break the hydrogen bonds that bind water molecules together. [27]

In biophysics, hydrophobic interactions are crucial for understanding the three-dimensional structure of proteins. The stacking of hydrophobic bases has been considered a major contributor to the stability of the DNA double helix. Hydrophobic interactions that promote DNA self-assembly exhibit stronger biological stability and contain hydrophobic domains for loading functional molecules.[27] The hydrophobic interaction is widely recognized as a primary catalyst for protein folding and plays a crucial role in maintaining the globular or binding structures of individual proteins, multiproteins, and protein-ligand systems.[28]

To calculate the affinity, or fitness, of the protein-ligand interaction, an empirical scoring function sums up the contributions of various individual components. The scoring function in AutoDock Vina is based on the contributions of intermolecular interactions such as steric, hydrophobic, and hydrogen, as well as the number of rotatable bond interactions. Each interaction contribution assigns a different value in the AutoDock Vina scoring function.[29-31]

Protein-ligand binding can only take place spontaneously when the system's free energy is negative. The strength of the negative ΔG determines the degree of protein-ligand stability; the more negative the ΔG, the more stable the protein-ligand complex. A drug's capacity to bind to a receptor is measured by its binding affinity. The stronger receptor-ligand interactions are correlated with lower binding affinities and vice versa.[26,32,33]

Compounds of the coumarin group (clauslactone E, dentatin, nordentatin, clausenidin) and the carbazole alkaloid group (7 - hydroxyheptaphylline, clausine E, 2,7 - dimethoxy - 9H - carbazole - 3 - carbaldehyde and 2,7 - dimethoxy - 9H - carbazole - 3 - carboxylic acid) showed strong binding to the EGFR receptor because they had a negative binding affinity with ΔG values varying from -6.8 to -8.3 kcal/mol. There are several possible explanations for this effect: (1) the resulting hydrophobic interactions between all ligands and proteins, (2) the ligands meet Lipinski's criteria, (3) the docking results show that all compounds are capable of obstructing binding sites by binding to critical amino acid residues.

The results of this study are consistent with several studies that investigated various cancer cell types in vitro. Carbazole alkaloids found in the roots of C. excavata were reported to have a cytotoxic effect on Hela cancer cells.[11] Similarly, coumarin has demonstrated cytotoxic effects on lung cancer (NCI-H187), breast cancer (MCF7), and oral cavity cancer (KB).[15]

Doxorubicin is a highly potent antineoplastic drug and is widely used in several types of cancer, but it has very toxic side effects, can damage DNA and cause cardiotoxicity.[34-36] Perhaps this is because doxorubicin does not meet the five criteria of Lipinski's rule. It has a molecular weight exceeding 500 mg, hydrogen binding acceptors 12 and Hydrogen binding donors 6 (Table 1).

Based on this, doxorubicin is, therefore, poorly absorbed and permeated throughout the body. Numerous studies have demonstrated that the high molecular weight and chemical structure of anticancer medications are associated with their toxicities and side effects, as they may cause several metabolites to interact with drug off-target networks.[37] Despite its extreme side effects, such as cardiotoxicity, doxorubicin remains widely used today due to its efficacy in cancer treatment.[38]

EGFR is one of a wide family of receptor tyrosine kinases that are frequently expressed and/or mutated in many human cancers and have an important role in increased signaling and cancer development. Therefore, restoring apoptotic activity by targeting EGFR is an effective strategy to inhibit the proliferation of cancer cells.[39] Many preclinical and clinical studies have investigated the therapeutic potential of agents targeting EGFR by inactivating or blocking it with various small molecules, both synthetic and natural compounds.[40,41]

In accordance with these findings, our in silico analysis showed that the natural compounds four coumarin compounds (clauslactone E, dentatin, nordentatin, Clausenidin) and four carbazole alkaloid compounds (7 - hydroxyheptaphylline, clausine E, 2,7-dimethoxy - 9H - carbazole - 3 - carbaldehyde, and 2,7 - dimethoxy - 9H - carbazole - 3 - carboxylic acid) have potential as ligands that inhibit the anti-apoptotic EGFR protein.

However, this molecular docking study has many limitations. First, molecular docking can only determine whether a compound has a strong bond or not with a molecule but cannot know the mechanism of action of the compound in detail. Another limitation of this research is its inability to determine the compound's dose in vitro or in vivo studies, which is crucial. Additionally, there are still discrepancies between virtual data derived from molecular docking and in vivo

experimental data, necessitating in vivo verification and integration with other experimental methods.

CONCLUSION

Four coumarin compounds (clauslactone E, dentatin, nordentatin, and clausenidin) and four carbazole alkaloid compounds (7 - hydroxyheptaphylline, clausine E, 2,7 - dimethoxy - 9H - carbazole - 3 - carbaldehyde, and 2,7 - dimethoxy - 9H - carbazole - 3 - carboxylic acid) are candidate ligands to restore apoptotic activity in cancer cells by acting through interaction with EGFR. The four coumarin compounds (clauslactone E, dentatin, nordentatin, and clausenidin) and four carbazole alkaloid compounds (7 - hydroxyheptaphylline, clausine E, 2,7 - dimethoxy - 9H - carbazole - 3 - carbaldehyde, and 2,7 - dimethoxy - 9H - carbazole - 3 - carboxylic acid) have the potential to be effective anti-cancer candidates by inhibiting EGFR in the apoptosis-targeting pathway. Implication of research is to validate the *in silico* results. Further *in vitro* and *in vivo* experiments are needed.

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Informed Consent Statement: The research was "Not applicable" for studies not involving humans and animals.
Data Availability Statement: In compliance with research ethics guidelines, all researchers will grant permission via email for availability and access to study data.
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