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Article

In Silico Evaluation of Bisbenzylisoquinoline Compounds from *Stephania* sp. as EGFR Inhibitors for Triple-Negative Breast Cancer

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Abstract. Triple-negative breast cancer (TNBC) represents a highly aggressive breast malignancy subtype characterized by constrained therapeutic alternatives and poor clinical outcomes. Since Epidermal Growth Factor Receptor (EGFR) is frequently overexpressed in TNBC, it remains a strategic molecular target for anticancer drug innovation. This study evaluates the inhibitory potential of bisbenzylisoquinoline (BBIQ) compounds from *Stephania* species against EGFR using an *in silico* framework. Molecular docking analysis revealed that isotrilobine possessed the highest binding affinity toward EGFR, achieving a docking score of -10.5 kcal/mol, indicating potent interactions within the active site. Molecular dynamics simulations over a 100 ns trajectory demonstrated that the EGFR-isotrilobine complex maintained structural stability throughout, as evidenced by RMSD values of 0.193-1.223 Å, RMSF of 1.5 ± 1.1 Å, radius of gyration (Rg) of 4.597-4.918 Å, and a solvent-accessible surface area (SASA) of 772.3 Å². Hydrogen bond analysis identified an average of four stable interactions, reinforcing persistent ligand binding within the receptor pocket. Furthermore, MM-PBSA calculations yielded a binding free energy of 101.063 ± 127.26 kJ/mol, indicating energetically favorable binding. These findings suggest that isotrilobine and related BBIQ analogues exhibit significant potential as EGFR inhibitors for TNBC therapy, serving as viable candidates for further experimental validation and drug development.

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1. Introduction

Breast cancer (BC) is the most common cancer among women. Breast cancer (BC) is a highly heterogeneous disease containing different BC molecular subtypes that, in general, are classified based on patterns of expression of cell surface receptors. Triple negative breast cancer (TNBC), is a subtype of breast neoplasm that lack estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) receptors on its cell surface [1]. TNBC accounts for 15–20% of diagnosed breast cancers and is generally more aggressive, having few treatment options and a poor prognosis than other subtypes [2]. Chemotherapy, radiation therapy, and surgery are the current methods of treatment. Even while TNBC is sensitive to traditional anticancer drugs, more than 70% of patients do not achieve a full response, which raises the death rate when compared to non-TNBC subtypes [3].

One of the molecular targets most studied is EGFR, which is significant for cell growth regulation and one of the most hotly pursued TK targets. A significant number of clinical cancers arise due to inappropriate activation of TKs through various mechanisms such as point mutations, despite the pivotal role they play in growth, division, metastasis and survival cells [4]. In TNBC, EGFR is often overexpressed and involved in promoting tumor growth, invasion and metastasis through downstream pathways including MAPK/ERK and PI3K/AKT [5-6]. Thus, EGFR is a potential therapeutic target for developing effective treatment strategies against TNBC.

Given the limitations of current therapeutic approaches, the exploration of natural products is gaining increasing attention as a potential source of new anticancer compounds. Natural compounds are known for their high structural diversity and their ability to target multiple molecular pathways simultaneously, making them particularly well-suited for complex diseases such as TNBC [7]. Natural products have made a significant contribution to the development of anticancer drugs, with more than half of approved drugs derived from or inspired by such sources [8]. One prominent class is alkaloids, which have been reported to inhibit cancer progression by targeting key processes such as proliferation, apoptosis, and metastasis [9].

Stephania (family Menispermaceae) has been widely explored by phytochemical screenings, revealing a high diversity of bisbenzylisoquinoline alkaloids (BBIQ), with broad pharmacological activities including anti-inflammatory and anticancer [10]. Numerous BBIQ molecules have shown promising anticancer effects in previous studies. For instance, Fangchinoline has been reported to display pronounced anticancer effects via suppression of cancer cells' multiplication as well as cell death induction through blocking specific signaling pathways including the EGFR-mediated PI3K/AKT route [11]. Similarly, tetrandrine with strong antiproliferative effects can also induce apoptotic cell death and inhibits tumor growth in breast cancer by modulating drug resistance-associated pathway and controlling cell cycle progress [12].

Computational approaches play a critical role in the modern drug discovery process and can be used to further our understanding of the molecular basis for anticancer activity. Molecular docking is a well-established method used for estimating binding affinity and the characterization of ligand–protein interactions profiles, while molecular dynamics (MD) simulations lead to evaluation of complex structural stability and dynamic behavior. These analyses include parameters like the root mean square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg) and solvent-accessible surface area (SASA), as well as hydrogen bonding interactions and binding free energy by Molecular Mechanics Poisson–Boltzmann Surface Area (MM-PBSA) [13-14]. However, grand studies targeting the EGFR using an integrated docking and MD simulation framework on bisbenzylisoquinoline alkaloids (BBIQ) from Stephania have been few. Thus, the purpose of this research was to study EGFR as a therapeutic target in TNBC by combined computational strategy against Stephania derived BBIQ compounds.

2. Experimental Section

2.1. Materials

The study included selection of eleven bisbenzylsoquinoline (BBIQ) alkaloids previously reported from Stephania: cepharanoline, cepharanthine, hypoepistephanine, oxyacanthine, stebisimine, fangchinoline, isotetrandrine, obamegine, (–) tetrandrine and (+)-tetrandrine. Computational analysis was carried out using high performance computer (HPC) with intel ® Xeon® CPU E5-2699 v3 processor and 128GB RAM. Software used included Cytoscape 3.10.3, Autodock Vina, PyMOL, BIOVIA Discovery Studio 2021 and YASARA 23.9.29 (License No: 673924518). The information and molecular data were acquired from public databases: Pubchem (https://pubchem.ncbi.nlm.nih.gov/), Protein Data Bank (RSCB PDB) (www.rcsb.org).

2.2. Ligands Preparation

Ligand structures were imported into AutoDock Tools 1.5.6 as an SDF file from the PubChem database with pre-determined three-dimensional (3D) conformations. The molecules were optimized using energy minimization. Which was followed by the incorporation of polar hydrogen atoms, Gasteiger charges were then assigned to each ligand. The rotatable bonds were defined in order to ensure more conformational flexibility, non-polar hydrogens were set up by using default settings. Ultimately, the ligands were prepared saved in PDBQT format for further docking simulations.

2.3. Protein Preparation

The three-dimensional structure of the epidermal growth factor receptor (EGFR) (PDB ID: 5UGC; resolution ≤ 2.00 Å) was retrieved from the RCSB Protein Data Bank [15]. The protein preparation was carried out using AutoDock Tools by removing the co-crystallized ligand and water molecules but preserving the structural components necessary for docking. Next, hydrogen atoms were added and protonation states were changed to physiological pH. Kollman charges were then added, and the structures converted to PDBQT format for docking. To facilitate further analysis, the kinase domain was retained.

2.4. Docking Validation

Docking validation was done by redocking the native co-crystallized ligand back into to the EGFR active site using AutoDock Vina. The accuracy was evaluated against the crystallographic structure for this comparison of predicted pose. Molecular docking simulations were performed to evaluate the ligand binding abundance toward EGFR and the RMSD ≤ 2.0 Å using PyMOL that validated the protocol used. The native ligand position was used to define a binding site using a grid box of 60 × 35 × 35 Å, while docking parameters were defined as an exhaustiveness value of 80 and up to a maximum of 10 binding modes. The generated poses were calculated by their binding affinities (kcal/mol) and ranked then the best conformations for interaction studies were chosen other software Discovery Studio [16-17].

2.5. Molecular Dynamics

Conformational stability and dynamic behavior of protein-ligan complexes were explored with the MD simulations. The AMBER14 force field model all system parameters and simulations were conducted using licensed YASARA software (license ID: 673924518). Simulation systems remained physiologically relevant (310 K and pH 7.4 with solvent by using TIP3P water model, included neutralized Na+ and Cl− articles intentionally to mimic the in vivo ionic force. MD simulations were then conducted at 1 atm 100 ns, time step 2 fs [18]. Trajectory analyses included RMSD, RMSF, Rg, SASA), and hydrogen bond interactions [19].

2.6. MM-PBSA Calculation

Trajectory analysis analyzed by default scripts from YASARA macros. Subsequently, snapshots obtained from these simulations were extracted and processed as inputs to binding free energy calculations based on the MM-PBSA (Molecular Mechanics/Poisson–Boltzmann Surface Area) method.

Binding free energy = E_potReceptor + E_solvReceptor + E_potLigand + E_solvLigand - E_potComplex - E_solvComplex

The AMBER14 force field was applied and calculations were performed using the built-in YASARA macros in MM-PBSA mode. The stability of the protein–ligand complexes was assessed by taking solvation effects into account using a Poisson–Boltzmann Surface Area (PBSA) model. For this method, higher binding free energy values are interpreted to mean greater ligand–protein binding affinity [20].

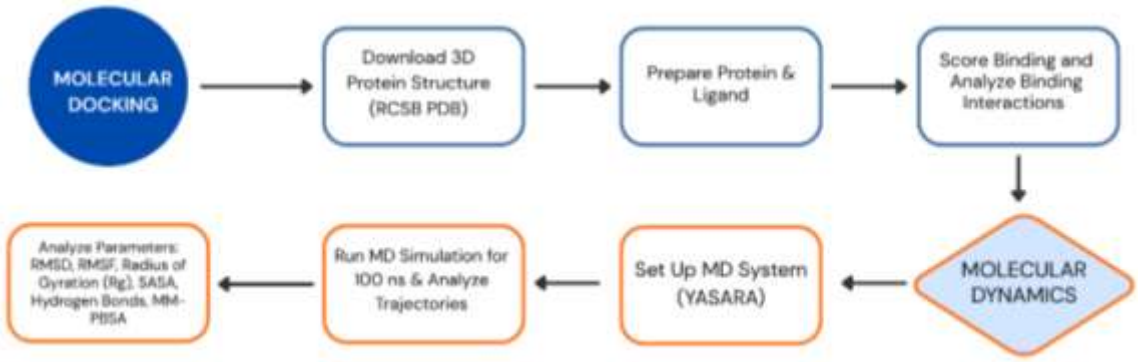


Figure 1. Research workflow diagram of molecular docking and molecular dynamics

3. Results and Discussion

3.1 Molecular Docking

Eleven compounds including the reference medication Lapatinib were used in molecular docking against EGFR (PDB ID: 5UGC) (Table 1). To verify the docking procedure, the co-crystallized ligand was redocked, producing an RMSD value of 0.854 Å for EGFR. This figure is lower than the generally accepted threshold of 2.0 Å, suggesting that the docking approach is valid and able to reliably recapitulate the native binding pose [21]. Lower values of docking energy indicate stronger binding affinity [22]. The EGFR target protein formed three complexes with the strongest binding affinities, namely isotrilobine (−10.5 kcal/mol), followed by cepharanoline (−9.1 kcal/mol) and cepharanthine (−9.1 kcal/mol). These results indicate that BBIQ compounds may possess strong binding potential toward EGFR, suggesting possible selectivity against triple-negative breast cancer (TNBC). This highlights their potential as targeted therapeutic candidates for the treatment of this aggressive cancer subtype.

Table 1. The docking affinity and interaction of compounds binding to the protein target

Compound	Binding Affinity (kcal/mol)
8BS (Native Ligand)	-8.7
Lapatinib	-9.6
Cepharanthine	-9.1
Fangchinoline	-9.0
Hypoepistephanine	-7.8
Isotetrandrine	-8.0
Obamegine	-8.1
(-)-Tetrandine	-8.9
(+)-Tetrandine	-8.9
Oxycanthine	-8.5
Isotrilobine	-10.5
Stebisimine	-8.6
Cepharanoline	-9.1

Figure 2. shows the diversity of EGFR receptor interacting residues in a 2D interaction profile for BBIQ. Isotrilobine exhibits the most extensive and comprehensive interactions with key EGFR residues, namely Ala743, Lys745, Ser744, Val726, Met793, Pro794, Phe795, Arg796, Asp800, Arg841, Leu844, Thr854, and Phe856. The involvement of Met793 indicates interaction at the hinge region, which is a crucial area in EGFR inhibition [23], while residues such as Lys745, Arg796, Arg841, and Asp800 contribute to polar and electrostatic interactions. In addition, hydrophobic residues including Val726, Leu844, Pro794, Phe795, and Phe856 enhance complex stability through hydrophobic interactions. This extensive interaction pattern suggests that isotrilobine is able to optimally occupy the ATP-binding site [4],[24].

Cepharanthine shows interactions with Arg841, Ala722, Phe856, Cys797, Asn842, Asp837, Phe723, Arg858, Pro877, and Lys875. Interactions with Cys797 and Arg841 indicate potential involvement in the active site of EGFR; however, the number of interacting residues is more limited compared to isotrilobine, resulting in a relatively simpler complex formation. Cepharanoline also exhibits a similar interaction pattern, involving Arg841, Ala722, Phe856, Lys875, Pro877, Arg858, Asp837, Phe723, Ser720, and Gly721. Although a considerable number of residues are involved, the dominance of surface and non-catalytic residues suggests that cepharanoline interacts mainly around the binding pocket without deep penetration into the catalytic core.

Lapatinib, as a reference drug, interacts with important residues such as Met793, Cys797, Arg841, Val726, Leu844, Phe856, Ala743, Pro794, Thr854, Asn842, and Gly721. The presence of Met793 and Cys797 indicates that lapatinib binds to the hinge region and the active site of EGFR, consistent with its mechanism as a tyrosine kinase inhibitor. Meanwhile, the native ligand interacts with Met793, Leu718, Ser720, Met790, Phe723, Leu792, Gly796, Pro794, Lys728, Ala743, Val726, Gln791, Leu844, Arg841, and Phe856. This pattern reflects the natural physiological interaction within EGFR, with strong involvement in the hinge region (Met793) and the ATP-binding site.Overall, isotrilobine demonstrates the most extensive and stable interactions with key EGFR residues, particularly Met793 and Cys797, which are essential for catalytic activity. This finding is consistent with previous docking results that position isotrilobine as the most promising EGFR inhibitor candidate

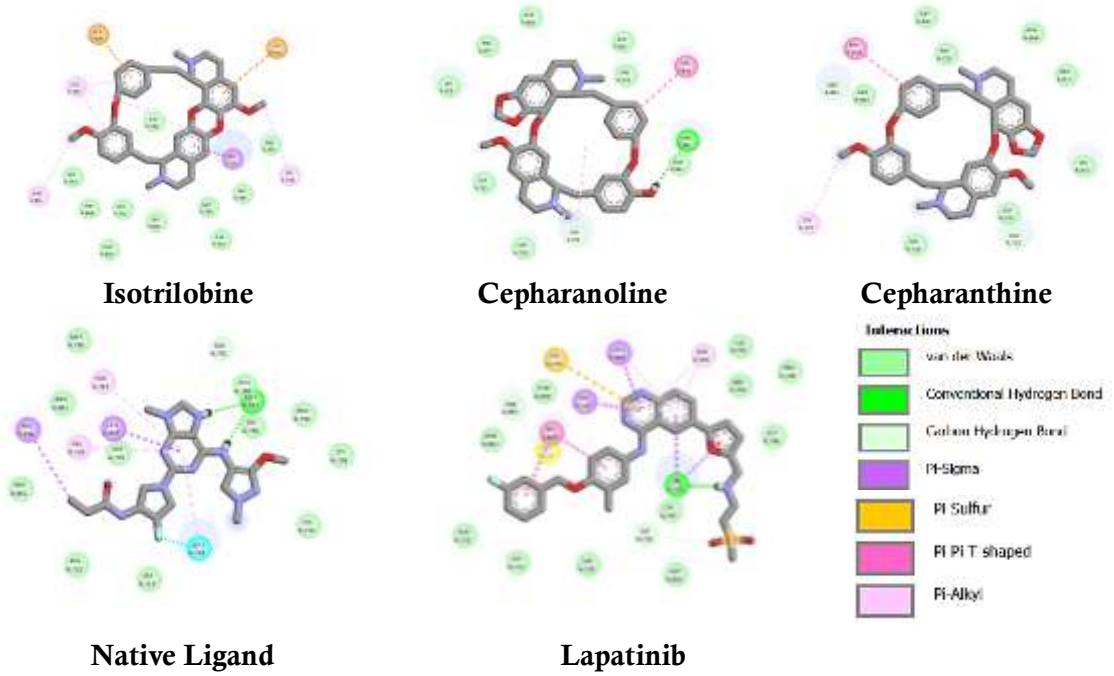


Figure 2. Molecular docking between the target protein EGFR

3.2 Molecular Dynamics

MD simulation is a computational approach to observe the biophysical interaction where structural changes and flexibility of docked complexes can be visualized during simulation time [24-26]. Using MD simulation, you can observe how a protein behaves and reshapes in a biological system. MD trajectory comparison is possible for RMSD, RMSF, Rg, SASA and H-bond and MM-PBSA [27].

The conformational stability of EGFR-Ros1 complexes containing the evaluated compounds was analyzed at the simulation time period by calculating RMSD of Cα atoms for each complex as compared to its respective initial structure [26]. It is also crucial that the complex remains stable, as this is a prerequisite for further investigations notably deviations in the range of 1–3 Å are acceptable [28]. The EGFR–Isotrilobine complex exhibited RMSD values ranging 0.193-1.223 Å, reflecting minimal structural deviation and indicating a highly stable complex with limited conformational changes. The EGFR–native ligand complex showed RMSD values between 0.922-2.558 Å, remaining within the acceptable range and indicating stable behavior throughout the simulation. Meanwhile, the EGFR–Lapatinib complex displayed RMSD values 0.813-3.763 Å, suggesting relatively higher fluctuations with some deviations exceeding the acceptable range, indicating greater conformational flexibility.

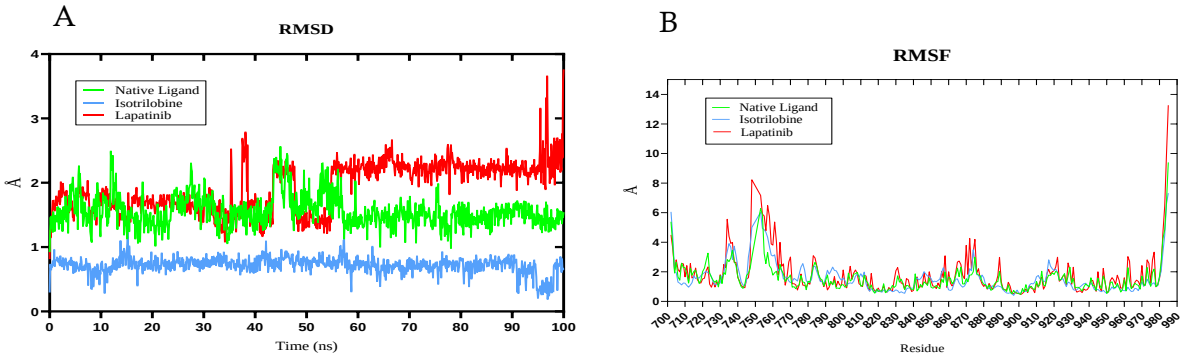
RMSF scores of protein backbone amino acids show remarkable changes at N- and C-terminals over the rest of the protein [29-30]. The structural movement and flexibility can be evaluated using RMSF of the residue. It is generally accepted that the binding site consists of few critical residues in the active pocket and tracking their movements could assist in elucidating protein−ligand interaction [31]. The RMSF values EGFR-Native ligand, EGFR-Isotrilobine, and EGFR-Lapatinib complexes are 1.7 ± 1.2, 1.5 ± 1.1, and 2.1 ± 1.6. This shows Figure 3B, the EGFR-Isotrilobine complexes have less residue movement and are more stable than the native ligand and drug.

Rg was employed to assess the compactness of simulated systems, which describes the proximity of atoms around center of mass. In this case, Rg was used to determine if known stable folded structures remain in a stable folded state or has undergone unfolding during the simulated periods of time [32]. The EGFR-Native ligand complex had a range of 4.411-4.824 Å, the EGFR-Isotrilobine

complex had a range of 4.597–4.918 Å, while the EGFR-Lapatinib complex showed the highest Rg value of 4.51–7.316 Å with significant fluctuations, indicating an unstable complex during the simulation. The simulation results for the EGFR-Lapatinib complex, showing significant fluctuations in Rg values 4.51–7.316 Å Figure 3C, indicate that the complex is unstable and likely undergoes greater conformational changes during the simulation. This corresponds to a looser or unfolded structure, reflected in the higher and fluctuating Rg values. In contrast, the EGFR-Isotrilobine and EGFR-native ligand complexes show more constant Rg values, indicating a more stable structure and stronger ligand interactions with the binding site [33].

The SASA is a crucial parameter to evaluate surface area of protein exposed to solvent molecules, so it provides information about the degree of conformational changes of the protein during interacting with compound [34]. In Figure 3D the SASA curve EGFR-Native ligand (675 Å²), EGFR-Isotrilobine (772.3 Å²), and EGFR-Lapatinib (742 Å²) fluctuated up to 885 Å², indicating that the EGFR-Lapatinib complex was unstable during the simulation. The research shows that SASA variations might signify conformational alterations in protein-ligand complexes, with elevated SASA values associated with enhanced surface exposure and conformational dynamics during molecular dynamics simulations [35]. SASA reflects the amount of surface area that is exposed and from a binding perspective, sustained low and stable SASA values are an indication of a ligand well nestled within the binding pocket with a proportionally limited solvent interaction. This behavior often implies that there were strong and stable binding contacts persisted during the course of the simulation [36].

Hydrogen bonds are key interactions that stabilize protein structure and facilitate protein–ligand binding. Disruption of one or more inter-protein hydrogen bonds is commonly linked to conformational changes in the protein complex [37]. In EGFR, the native ligand formed 6, isotrilobine formed 4, lapatinib formed 9 Figure 3E. Although lapatinib exhibited a higher number of hydrogen bonds, isotrilobine showed more consistent hydrogen bond interactions throughout the simulation, indicating higher hydrogen bond occupancy and longer interaction persistence. It is well known that stability of protein-ligand interactions relies not only on the total number of hydrogen bonds but also on their occupancy and residence time [38]. MM-PBSA has been extensively used to estimate ligand–receptor binding free energy and it accounts for the predominant interaction contributions in a complex [39]. The EGFR-Native ligand complex had a value of 193.39 ± 123.22 kJ/mol, EGFR-Isotrilobine 101.063 ± 127.26 kJ/mol, while EGFR-Lapatinib 148.44 ± 125.36 kJ/mol Figure 3F. Overall, the native ligand, isotrilobine, and lapatinib complexes maintained stable interactions and consistent behavior throughout the simulation in accordance with physical principles [40].



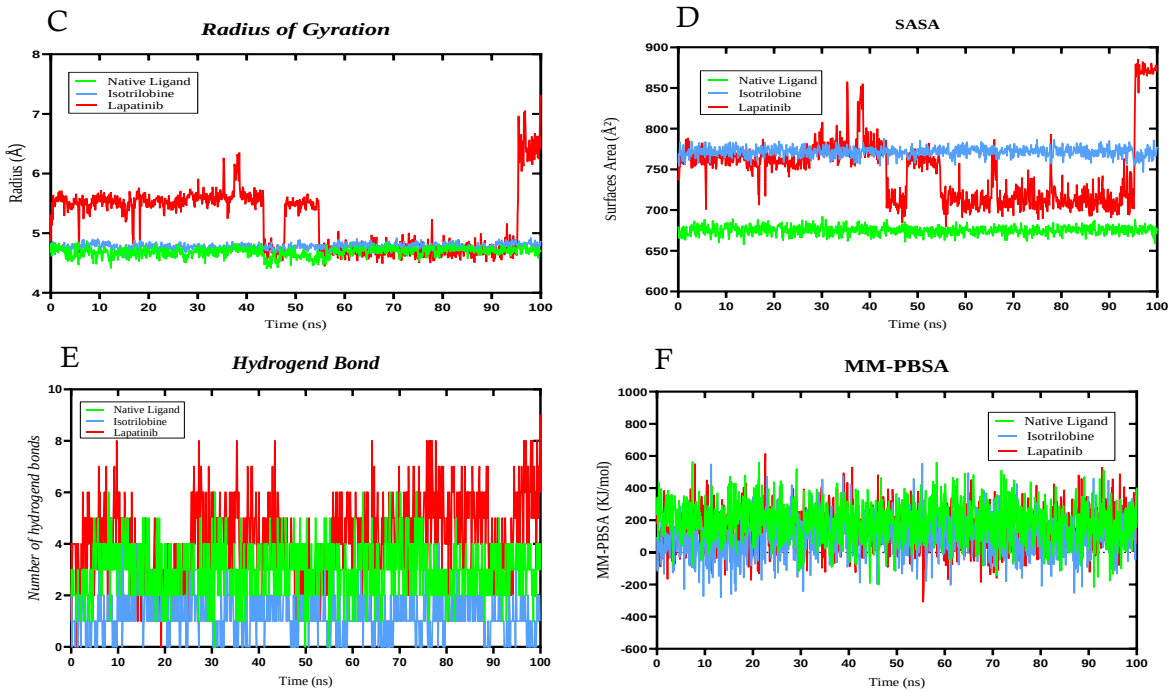


Figure 3. (a) RMSD, (b) RMSF, (c) Rg, (d) SASA, (e) H-bond, (f) MMPBSA of lapatinib (red), isotrilobine (green), and native ligand (blue) with EGFR, showing the structural stability and fluctuation of each complex during the 100 ns simulation.

4. Conclusion

In silico analysis yielded promising results where isotrilobine and other Bisbenzylisochinoline (BBIQ) compounds derived from Stephania showed a potential to act as EGFR inhibitors in TNBC. MD predicts a stable EGFR–isotrilobin complex based on RMSD, RMSF, Rg and SASA values having small ranges along with relatively constant hydrogen bonding interactions over time whereas molecular docking proves its binding affinity as well as intensified interactions among complexed residues in the active site of the protein. Moreover, the conservation of high binding energy is further confirmed with MM-PBSA analysis. These findings indicate that isotrilobine may be a potential EGFR inhibitor and thus may be effective in the treatment of TNBC; further in vitro and in vivo studies are needed to confirm these data.

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