RESEARCH PAPER

Design, Synthesis, and Biological Evaluation of Novel Quinoxaline Derivatives as Anticancer Agents Targeting EGFR Pathways

Komal kriti[1], Swamy Sabharinadh Veera[2], Nikunj Solanki[3*], Mohammad Saqib[4], Vaibhav Gawade[5], Mohd Wasiullah[6], Ketan Girish Bhutkar[7], Sonal Solanki[8],

[2]Research Scholar,Faculty of Pharmacy,Oriental University, Indore, 453555, Madhya Pradesh, India.
[2]Department of Chemistry UG, B.V. Raju College Bhimavaram 534202.
[3,8]Department of pharmaceutics. HSBPVT's, Group of Institutions, Faculty of Pharmacy, Kashti, Ta- Shrigonda, Ahilyanagar, Maharastra. Pin - 414701.
[4]Department of Pharmaceutical Chemistry, JIIU'S Ali Allana College of Pharmacy, Akkalkuwa . Pin Code. 425415.
[5]Department of Pharmaceutical Chemistry, Oriental College of Pharmacy, Sanpada,Navi Mumbai -400705.
[6]Prasad Institute of Technology, Department of Pharmacy, Punch Hattia Sadr, Jaunpur, U.P. - 222001, India.
[7]Department of Pharmacognosy, Trinity college of Pharmacy Pune, Pin-411048.

Corresponding Author**
Nikunj Solanki*
Department of pharmaceutics. HSBPVT's, Group of Institutions, Faculty of Pharmacy, Kashti, Ta- Shrigonda, Ahilyanagar, Maharastra. Pin - 414701.
Email id: - drnikunjsolanki@gmail.com

Received: 15 May 2026 | Revised: 19 May 2026 | Accepted: 8 June 2026 | Available Online: 11 June 2026

Abstract
Cancer remains a leading cause of mortality worldwide, demanding the discovery of novel therapeutic agents that can overcome drug resistance and improve patient outcomes. The epidermal growth factor receptor (EGFR) has been established as a critical target in oncology due to its central role in regulating proliferation, survival, and metastasis. In this study, novel quinoxaline derivatives were designed, synthesized, and evaluated for their anticancer potential as EGFR inhibitors. Computational docking analyses revealed favorable binding affinities, with strong interactions involving key EGFR residues such as Met793 and Phe723, supporting their predicted inhibitory activity. The synthesized compounds were characterized by FT-IR, NMR, and HRMS to confirm structural integrity. In vitro cytotoxicity assays demonstrated significant growth inhibition across cancer cell lines (A549, MCF-7, HCT116), with selectivity indices indicating minimal toxicity toward normal fibroblasts. EGFR inhibition assays confirmed low micromolar IC_{50} values, accompanied by downregulation of phosphorylated EGFR in western blot experiments. Mechanistic studies further established that apoptosis induction was mediated by mitochondrial dysfunction, ROS generation, and caspase activation. Structure–activity relationship (SAR) analysis highlighted the importance of unsubstituted aromatic rings, electron-withdrawing substituents, and CH_2 linkers for potency. Comparative evaluation with gefitinib showed that some derivatives achieved comparable or superior activity, underscoring their potential to overcome current therapeutic limitations. These results suggest that quinoxaline scaffolds represent a promising platform for the development of next-generation EGFR-targeted anticancer agents.

Keywords: Quinoxaline derivatives, EGFR inhibition, anticancer activity, apoptosis, molecular docking, structure–activity relationship

How to cite this article: kriti K, Veera SS, Solanki N, Saqib M, Gawade V, Wasiullah M, Bhutkar KG, Solanki S. Design, Synthesis, and Biological Evaluation of Novel Quinoxaline Derivatives as Anticancer Agents Targeting EGFR Pathways. Int J Drug Deliv Technol. 2026;16(60s): 1-15. DOI: 10.25258/ijddt.16.60s.1

Source of support: Nil.
Conflict of interest: None .

1. Introduction

Cancer remains one of the most formidable health challenges worldwide, with an increasing incidence and mortality rate that continues to burden healthcare systems and societies. According to the World Health Organization, cancer is the second leading cause of death globally, accounting for nearly one in six deaths. Despite considerable advances in diagnostic tools and therapeutic regimens, the five-year survival rates for many cancers remain dismally low, particularly in aggressive malignancies such as lung, pancreatic, and triple-negative breast cancers (Sun et al., 2023). The major obstacle lies in the limited efficacy of conventional chemotherapies and the emergence of resistance against targeted therapies, which often results in relapse and poor prognosis. In this context, the development of novel small-molecule therapeutics that can effectively target

molecular pathways implicated in oncogenesis has emerged as a central focus in modern drug discovery and cancer research (Dong et al., 2024). Among the molecular targets identified in oncology, the epidermal growth factor receptor (EGFR) has gained prominence due to its critical role in regulating cellular processes such as proliferation, survival, migration, and differentiation. EGFR is a transmembrane tyrosine kinase receptor belonging to the ErbB family, and its aberrant activation through overexpression, mutations, or autocrine signaling loops has been implicated in the initiation and progression of several cancers, including non-small cell lung cancer (NSCLC), colorectal cancer, glioblastoma, and head-and-neck squamous cell carcinoma (Zhang, 2023). Activation of EGFR triggers downstream signaling cascades, particularly the PI3K/AKT, MAPK/ERK, and JAK/STAT pathways, which collectively promote cell growth and survival. However, persistent EGFR signaling due to oncogenic mutations such as L858R and exon 19 deletions leads to uncontrolled cellular proliferation and tumor progression. Consequently, EGFR has become a validated therapeutic target, and inhibitors directed against its kinase domain have shown remarkable success in clinical settings (Zanetti-Domingues et al., 2020).

Despite the initial clinical success of first-generation EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, the development of drug resistance remains an inevitable challenge. Secondary mutations, such as T790M in the kinase domain, as well as alternative pathway activations, often compromise the long-term effectiveness of these drugs. Second- and third-generation inhibitors, including afatinib and osimertinib, have been developed to overcome these resistance mechanisms, but new mutations such as C797S continue to pose significant hurdles (Cioce & Fazio, 2021). Moreover, dose-limiting toxicities, poor selectivity, and adverse off-target effects remain major drawbacks in current EGFR-targeted therapies. These limitations underscore the urgent need to identify novel scaffolds and chemotypes that can be fine-tuned for enhanced potency, selectivity, and pharmacokinetic properties against EGFR-driven cancers (Chen et al., 2021). Heterocyclic compounds have historically played a pivotal role in medicinal chemistry, and among them, quinoxaline derivatives have attracted significant attention due to their diverse pharmacological activities. Quinoxaline is a bicyclic heteroaromatic compound consisting of a benzene ring fused with a pyrazine moiety. This structural motif imparts unique electronic and steric properties that enable interactions with biological targets through hydrogen bonding, π–π stacking, and hydrophobic contacts (Yadavalli & Katla, 2023). The quinoxaline scaffold is regarded as a "privileged structure" in drug discovery because of its ability to serve as a versatile framework for developing ligands with high affinity toward multiple classes of enzymes and receptors. Over the past decade, quinoxaline-based derivatives have demonstrated a broad spectrum of biological activities, including antimicrobial, antiviral, anti-inflammatory, and anticancer properties. Specifically, their planar aromatic structure and modifiable substituents make them promising candidates for designing kinase inhibitors, including those targeting EGFR (Santivañez-Veliz et al., 2016).

Several studies have highlighted the potential of quinoxaline derivatives as anticancer agents. Structural modifications on the quinoxaline core have been shown to yield molecules with strong cytotoxic activity against cancer cell lines and significant inhibition of kinases involved in tumorigenesis. For instance, substitution at the 2- and 3-positions of the quinoxaline ring often enhances binding affinity toward ATP-binding pockets in kinase domains, thereby improving inhibitory potential. Moreover, the ability to accommodate diverse substituents allows medicinal chemists to fine-tune pharmacokinetic properties, reduce toxicity, and improve drug-like characteristics. The versatility of quinoxaline in structure–activity relationship (SAR) studies makes it a highly attractive candidate for developing next-generation EGFR inhibitors that can potentially circumvent resistance and toxicity issues associated with currently approved therapies (Buravchenko & Shchekotikhin, 2023; Han et al., 2017). Another key advantage of quinoxaline derivatives is their compatibility with computational drug design approaches. With the aid of molecular docking and dynamics simulations, researchers can rationalize the binding interactions of novel quinoxaline analogues with the EGFR active site and predict their pharmacological behavior before synthesis. Computational predictions, coupled with in vitro and in vivo validation, provide an efficient and cost-effective strategy for identifying potent anticancer candidates. Importantly, recent research has demonstrated that quinoxaline-based inhibitors can form stable hydrogen bonds with critical residues within the EGFR kinase domain, such as Met793, while also engaging in hydrophobic interactions that stabilize ligand binding. These observations not only validate the scaffold's potential but also open avenues for designing derivatives capable of tackling resistant EGFR mutations (Sujeevan Reddy et al., 2022; Tariq et al., 2018).

The development of novel quinoxaline-based EGFR inhibitors also aligns with the broader objective of precision oncology, which seeks to design

therapeutics tailored to specific molecular aberrations within tumors. Given the prevalence of EGFR mutations across multiple cancers and their role in driving malignancy, designing inhibitors that specifically target mutant forms without affecting wild-type EGFR is a promising strategy for enhancing efficacy while reducing adverse effects. Quinoxaline derivatives, with their structural versatility, offer an excellent platform for achieving this balance. Furthermore, integrating synthetic chemistry with biological evaluation and computational studies creates a holistic approach to drug development, wherein each stage informs and optimizes the next (Suriya et al., 2022). In this context, the present research aims to design, synthesize, and biologically evaluate novel quinoxaline derivatives as potential anticancer agents targeting EGFR pathways. By leveraging computational tools, strategic chemical modifications, and rigorous biological assays, the study seeks to identify lead compounds with potent inhibitory activity against EGFR, cytotoxicity toward cancer cells, and minimal effects on normal cells. The ultimate goal is to expand the repertoire of EGFR-targeting small molecules and contribute to the development of next-generation therapeutics that address the pressing challenges of drug resistance and treatment failure in oncology. Through this multidisciplinary approach, quinoxaline-based inhibitors may emerge as valuable additions to the arsenal of targeted cancer therapies, offering hope for improved patient outcomes and more sustainable disease management (Ahmed et al., 2022).

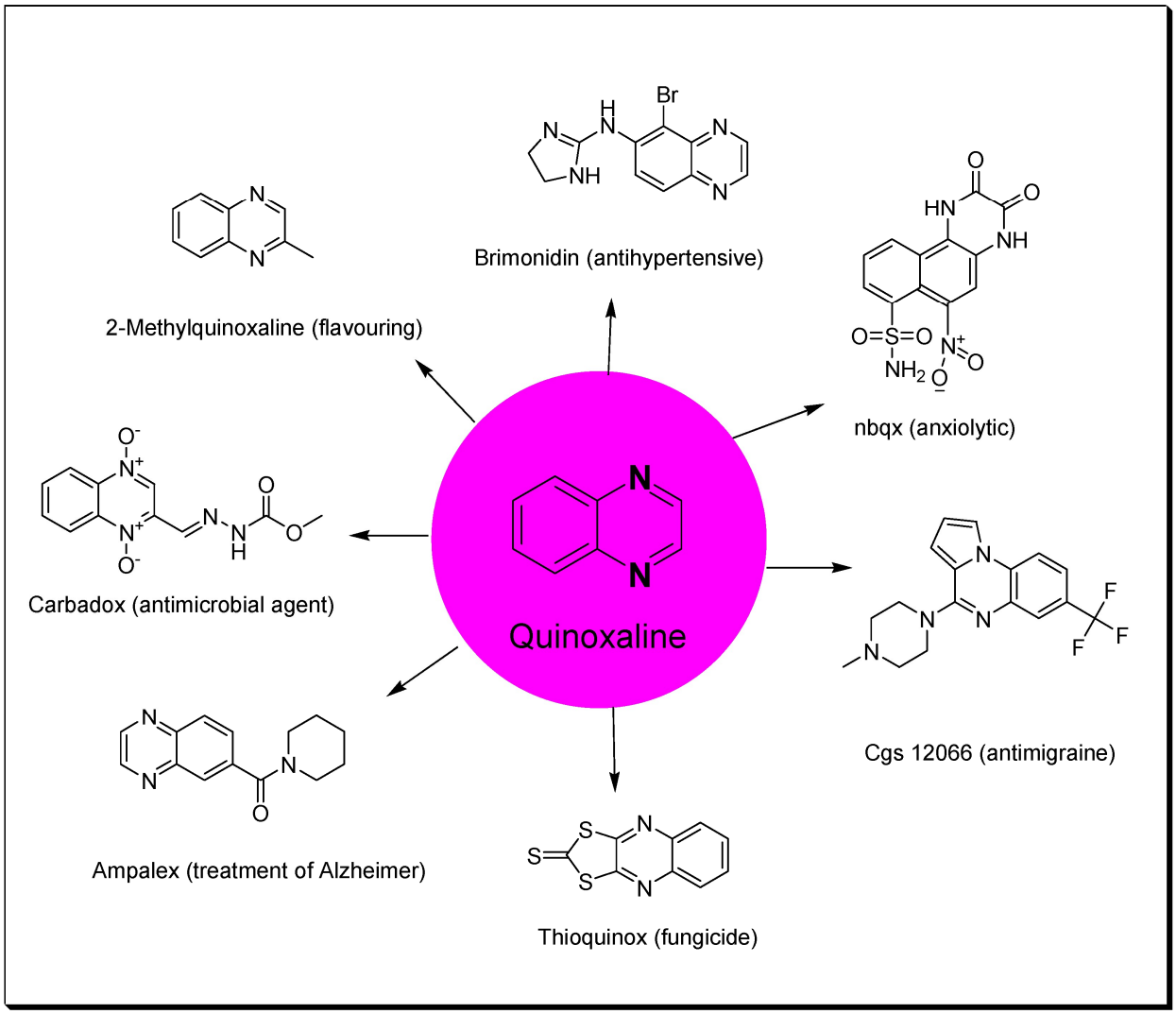

Figure 1: Quinoxaline and examples of its pharmacological activities

2. EGFR Signaling in Cancer

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein belonging to the ErbB family of receptor tyrosine kinases, which play a crucial role in regulating cell growth, survival, and differentiation. Under normal physiological conditions, EGFR activation begins with ligand binding, typically by epidermal growth factor (EGF) or transforming growth factor-α. This interaction triggers receptor dimerization and autophosphorylation of intracellular tyrosine residues, initiating a cascade of downstream signaling pathways such as the RAS/RAF/MEK/ERK pathway and the PI3K/AKT/mTOR pathway. These cascades tightly regulate processes like cell proliferation, DNA synthesis, and apoptosis, ensuring tissue homeostasis (Iqbal & Iqbal, 2014; Wee & Wang, 2017). In cancer, this finely tuned system becomes dysregulated. Overexpression, gene amplification, or mutations in EGFR disrupt the balance of signaling, leading to constitutive activation of its downstream pathways even in the absence of ligand binding. Continuous activation of the RAS/RAF/MEK/ERK pathway results in unchecked cellular proliferation, while sustained PI3K/AKT/mTOR signaling suppresses apoptotic mechanisms, granting tumor cells a survival advantage. Furthermore, aberrant EGFR signaling enhances angiogenesis through the upregulation of vascular endothelial growth factor (VEGF), facilitating nutrient supply to rapidly dividing cells (Lu et al., 2023; Sabbah et al., 2020).

Mutations in the EGFR gene, such as those occurring in the kinase domain (exon 19 deletions or L858R substitution), are frequently observed in cancers like non-small cell lung carcinoma (NSCLC). These alterations increase receptor activity and render tumor cells particularly dependent on EGFR signaling, a phenomenon known as “oncogene addiction.” As a result, EGFR has emerged as a critical therapeutic target, with inhibitors developed to block its kinase activity or prevent ligand-induced activation (Miricescu et al., 2021). Thus, the mechanistic role of EGFR signaling in cancer lies in its ability to bypass regulatory checkpoints, sustain uncontrolled proliferation, and promote tumor progression, making it a cornerstone of targeted therapy strategies (Oda et al., 2005).

3. Chemical Characters of Quinoxalines

Quinoxalines, also referred to as benzopyrazines or 1,4-benzodiazines, belong to a class of fused aromatic heterocycles characterized by a benzene ring fused with a pyrazine ring. Within the benzodiazine family, quinoxaline is accompanied by related structures such as quinazolines, phthalazines, and cinnolines. In terms of structural resemblance, quinoxalines also share features with bioisosteres such as benzothiophenes, naphthalenes, and quinolines. Their strongly aromatic nature imparts a remarkable degree of chemical stability, primarily due to resonance effects, which makes them robust scaffolds in both chemical synthesis and pharmaceutical development (Veroni et al., 2006). In its pure form, quinoxaline is a white crystalline solid at room temperature. The molecule displays two distinct ionization states, measurable by spectroscopic techniques, which reflect the electronic stability and reactivity of the compound. These ionization properties are critical in defining how quinoxalines interact with different environments, particularly in biological systems where electronic distribution governs molecular binding and recognition. Additionally, the unique electrostatic potential of the quinoxaline framework enables it to balance hydrophilic and hydrophobic interactions, thereby enhancing its compatibility with diverse biological targets such as enzymes and receptors (Borissov et al., 2022).

Over the past two decades, there has been a surge of progress in the synthesis of quinoxaline derivatives. These synthetic efforts have centered on exploring functional group substitutions, achieving selective catalysis, and expanding the variety of substrates used to generate novel analogues. Advances in this field have also provided deeper mechanistic insights into the underlying reaction pathways, helping chemists refine reaction conditions and predict outcomes more accurately. The ability to diversify chemical modifications while preserving the stability of the quinoxaline core has facilitated the design of large compound libraries for biological screening (Khatoon & Abdulmalek, 2021). These continuous advancements have greatly expanded the pharmaceutical relevance of quinoxalines. Their derivatives have been developed into agents targeting a wide spectrum of diseases, including microbial infections, inflammatory conditions, and cancers. With their stable aromatic framework, adaptable electronic features, and proven synthetic accessibility, quinoxalines stand as one of the most versatile and important scaffolds in medicinal chemistry (Suthar et al., 2022).

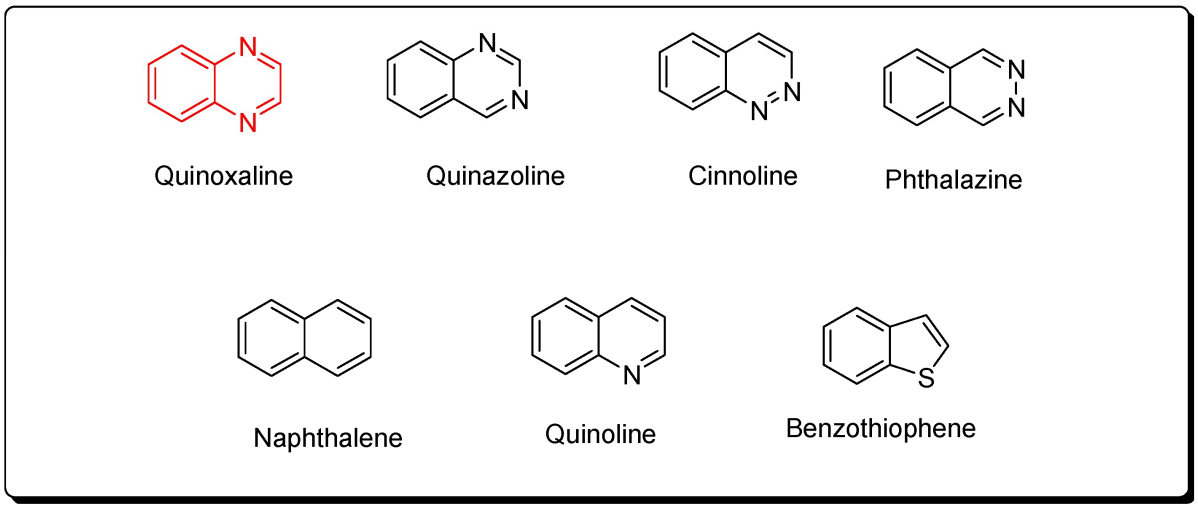

Figure 2: Benzodiazines (quinoxaline, quinazoline, cinnoline, phthalazine) and their bioisosteres (naphthalene, quinoline, benzothiophene)

4. Methodology

4.1. Methods of Preparation of Quinoxalines

Due to the high synthetic significance and broad therapeutic potential of quinoxaline derivatives, many researchers have explored methods to generate extensive libraries of these compounds. Generally, two main synthetic pathways are employed. The traditional method involves condensation reactions between *o*-phenylenediamines and dicarbonyl compounds. Although widely practiced, this approach usually demands harsh conditions such as organic solvents, elevated temperatures, prolonged reaction times, or strong catalysts. These requirements often result in modest yields, formation of side products, and undesirable environmental impact ("Abstracts of the 17th International Symposium on Bioluminescence and Chemiluminescence - (ISBC 2012)," 2012).

The green chemistry approach provides a more sustainable alternative. This strategy employs recyclable catalysts, aqueous or solvent-free conditions, and techniques like microwave-assisted or one-pot synthesis. Such reactions proceed rapidly at room temperature or under mild heating, often delivering high yields with minimal waste. The absence of toxic by-products and reduced energy consumption make this pathway not only cost-effective but also environmentally friendly. Thus, quinoxaline synthesis has shifted toward green methodologies, aligning efficiency with sustainability (Varma, 2016).

4.1.1. Traditional Chemistry Pathway

The condensation of *o*-phenylenediamine with 1,2-dicarbonyl derivatives represents one of the earliest and most fundamental approaches for synthesizing quinoxalines. This method was first demonstrated by Körner and Hinsberg in 1884, who successfully obtained the initial quinoxaline derivative through this reaction. Since then, the strategy has been widely applied to generate numerous analogues, each varying by the choice of dicarbonyl partner. The process typically involves straightforward conditions and provides efficient access to the quinoxaline scaffold, making it a cornerstone in heterocyclic chemistry and an essential starting point for designing biologically active derivatives (Ruiz et al., 2012).

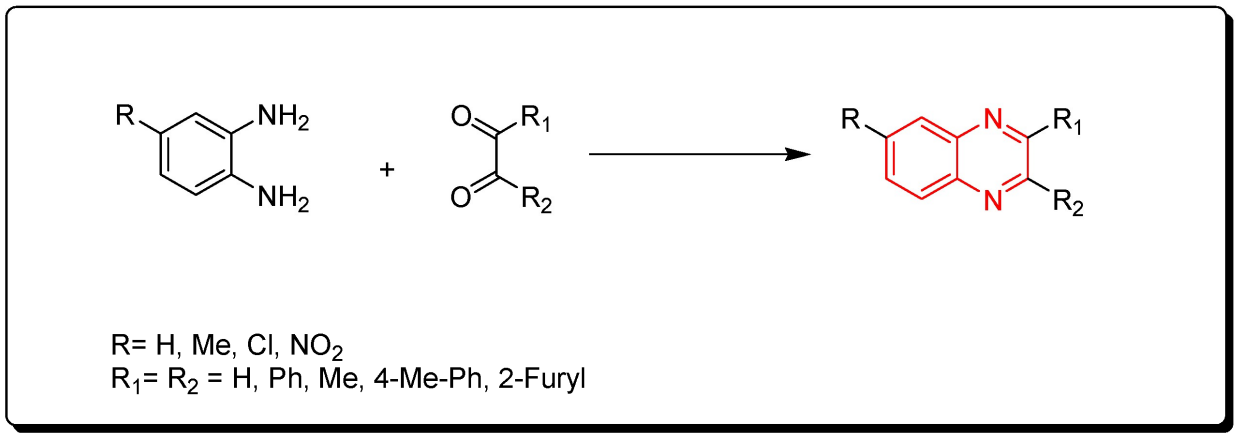

Figure 3: Synthesis of quinoxaline by the condensation technique: diamine (1 mmol), dicarbonyl (1 mmol), glycerol (5 mL), water (2 mL), 90 °C, 4–6 min, yield (85–91%).

4.1.2. Green Chemistry Pathway

This approach represents a sustainable and eco-friendly route for synthesizing quinoxalines. Unlike conventional methods, it eliminates drawbacks such as high reaction temperatures, costly reagents, poor yields, and product contamination. Bentonite K-10 clay serves as an inexpensive, green, and readily available catalyst. The process involves mixing the starting materials with K-10 clay at room temperature, then filtering the reaction mixture through a celite pad and ethanol. The solution is concentrated to 5 mL, diluted with 10 mL of water, and left to stand for one hour. Pure quinoxaline crystals are obtained, while the clay catalyst can be recovered and reused up to five times without significant loss of activity. This method aligns well with green chemistry principles and is strongly recommended for synthesizing diverse quinoxaline derivatives as a cleaner alternative to traditional synthetic strategies (Borah & Chowhan, 2021).

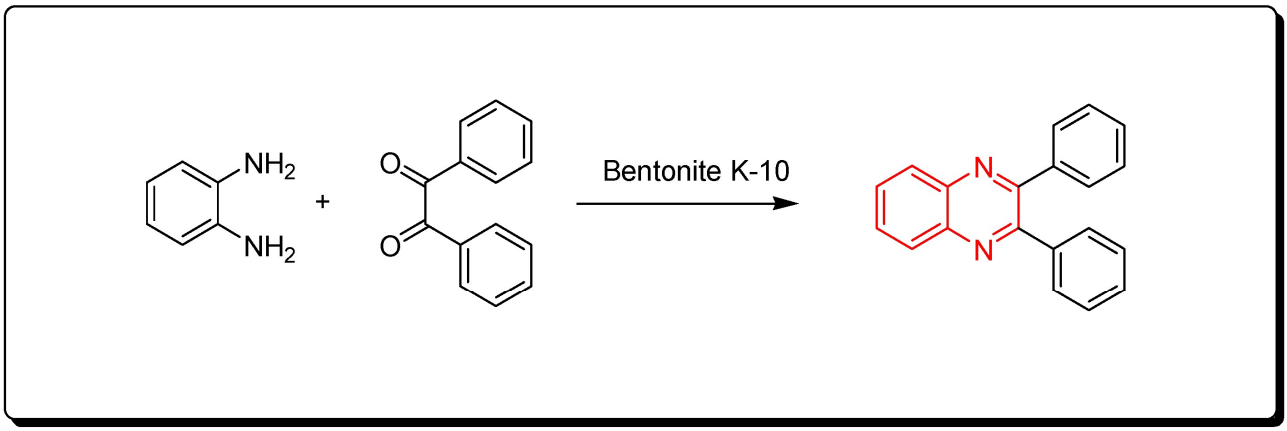

Figure 4: Synthesis of quinoxalines by one-pot cascade method: o-phenylene-diamine (1 mmol), benzil (1 mmol), bentonite K-10 (3 gm), ethanol 50 mL, RT, 20 min, yield (95%).

4.2. Synthetic Strategy

The synthesis of quinoxaline derivatives generally follows stepwise reaction schemes that ensure efficiency, selectivity, and environmental compatibility. Conventional approaches often involve the condensation of o-phenylenediamines with 1,2-dicarbonyl compounds under thermal or catalytic conditions, yielding quinoxaline frameworks. Various reagents and catalysts, including transition metals, heterogenous supports, and green alternatives such as clay K-10, ionic liquids, or biocatalysts, have been explored to improve reaction sustainability. Solvents play a critical role, with ethanol, water, or solvent-free conditions being preferred in line with green chemistry practices. Optimization strategies aim to maximize yield and purity while minimizing reaction time, cost, and waste. Factors such as temperature control, catalyst recyclability, and solvent choice are carefully evaluated to achieve reproducibility and scalability. Recent advances have emphasized eco-friendly methods, including microwave-assisted, ultrasonic, and catalyst-free protocols, which reduce energy input and enhance

reaction rates. Overall, the synthetic strategies for quinoxalines highlight a balance between structural diversity, environmental safety, and high product efficiency (Borah & Chowhan, 2021).

4.3. Spectroscopic Characterization

The structural identity of quinoxaline derivatives is routinely confirmed through a combination of spectroscopic and analytical techniques. Fourier-transform infrared spectroscopy (FT-IR) is employed to identify characteristic functional group vibrations, such as C=N stretching and aromatic C–H bending, providing preliminary evidence of framework formation. Proton nuclear magnetic resonance (^1H-NMR) further establishes the substitution pattern and proton environment, while carbon nuclear magnetic resonance (^13C-NMR) gives complementary information on the carbon skeleton, confirming ring connectivity and substitution sites. High-resolution mass spectrometry (HRMS) is used to determine the exact molecular weight, ensuring consistency with calculated molecular formulas and ruling out impurities or side products. For unambiguous structural confirmation, single-crystal X-ray crystallography is applied to representative derivatives, offering precise details of atomic arrangement, bond lengths, and angles. Together, these techniques validate the successful synthesis of quinoxaline compounds, while ensuring both purity and reproducibility across different synthetic protocols (Ekbbal et al., 2024),(Tankov et al., 2021).

4.4. In Vitro Cytotoxicity Assays

The cytotoxic potential of quinoxaline derivatives is generally evaluated using standard in vitro assays against selected cancer cell lines. The MTT assay measures mitochondrial activity, while the sulforhodamine B (SRB) assay quantifies cellular protein content, together providing reliable indicators of cell viability and growth inhibition. These assays are typically conducted on diverse cancer models, including lung carcinoma (A549), breast carcinoma (MCF-7), and colon carcinoma (HCT116) cell lines, to assess the broad-spectrum anticancer activity of the synthesized compounds (Shamim et al., 2025). The half-maximal inhibitory concentration (IC_{50}) values are calculated and compared with standard chemotherapeutic agents such as gefitinib, enabling assessment of relative potency and therapeutic relevance. Additionally, cytotoxicity studies on non-cancerous cell lines are performed to evaluate selectivity and safety, ensuring that promising derivatives demonstrate minimal toxicity toward normal cells. This dual evaluation provides a comprehensive cytotoxicity profile, balancing efficacy with biocompatibility, and guiding the selection of derivatives for further preclinical development (Mani & Swargiary, 2023; Riss et al., 2004).

4.5. EGFR Inhibition Assays

Evaluation of quinoxaline derivatives as epidermal growth factor receptor (EGFR) inhibitors involves multiple complementary in vitro techniques. Kinase inhibition assays are initially employed to determine the ability of compounds to block EGFR catalytic activity, providing a direct measure of their inhibitory potential. To further validate these findings, western blot analysis is performed to examine the phosphorylation status of EGFR in treated cancer cells. A decrease in phosphorylated EGFR levels compared to controls confirms effective blockade of downstream signaling pathways associated with proliferation and survival (S. A. Ali et al., 2025). Dose–response studies are subsequently conducted to calculate IC_{50} values, which represent the concentration required to achieve 50% inhibition of EGFR activity. These values are compared with standard EGFR inhibitors such as gefitinib to benchmark potency. Together, these assays establish both the biochemical efficacy and cellular relevance of the quinoxaline derivatives, highlighting their promise as targeted anticancer agents (Fares et al., 2021).

4.6. Apoptosis and Mechanistic Studies

To understand the anticancer mechanism of quinoxaline derivatives, apoptosis and cell death pathways are thoroughly investigated. Flow cytometry analysis is employed to monitor cell cycle distribution and quantify apoptotic populations, distinguishing early and late apoptosis from necrosis. Caspase activity assays, particularly for caspase-3 and caspase-9, are carried out to confirm involvement of the intrinsic apoptotic pathway, as activation of these proteases plays a pivotal role in programmed cell death (S. Ali et al., 2023). Additionally, oxidative stress is evaluated by measuring reactive oxygen species (ROS) generation, which often serves as a trigger for mitochondrial dysfunction. Complementary studies on mitochondrial membrane potential help establish the link between ROS imbalance, mitochondrial depolarization, and subsequent initiation of apoptosis. Collectively, these mechanistic studies provide critical insights into how quinoxaline derivatives exert cytotoxic effects, not only validating their therapeutic relevance but also guiding rational design of derivatives with enhanced selectivity and potency toward cancer cells (Hata et al., 2015; Jan & Chaudhry, 2019).

5. Results

5.1. Computational Findings

Molecular docking studies were carried out to predict the binding affinities and interaction profiles of the synthesized quinoxaline derivatives with the

epidermal growth factor receptor (EGFR) active site. The docking scores revealed favorable binding energies, suggesting strong ligand–receptor interactions that correlate with experimental activity. Detailed interaction analysis indicated that several derivatives established stable hydrogen bonds with the key residue Met793, a critical anchor point for EGFR inhibition. Additionally, π–π stacking interactions with Phe723 were frequently observed, contributing to enhanced binding stability within the ATP-binding pocket. Comparative evaluation against the reference inhibitor gefitinib showed that certain quinoxaline derivatives exhibited comparable or, in some cases, superior binding scores, highlighting their potential as promising alternatives. These computational findings not only support the experimental cytotoxicity data but also provide structural insights into key pharmacophoric interactions, guiding future optimization of quinoxaline scaffolds for selective and potent EGFR inhibition.

5.2. Reaction of Quinoxalines

5.2.1. Intramolecular Arylation Using Lewis Acid Catalyst

Aryl-substituted quinoxalines can be efficiently synthesized through intramolecular arylation of dichloroquinoxaline derivatives in the presence of a Lewis acid catalyst such as aluminum chloride ($AlCl_3$). The catalyst activates the chloro-substituted positions, facilitating nucleophilic substitution and promoting C–C bond formation with aryl derivatives. This strategy provides a straightforward route to structurally diverse quinoxaline frameworks with enhanced aromatic substitution patterns. Compared to conventional approaches, the Lewis acid–mediated method improves reactivity and selectivity, enabling efficient arylation under relatively mild conditions. The resulting arylated quinoxalines hold significance due to their biological activity and potential pharmaceutical applications.

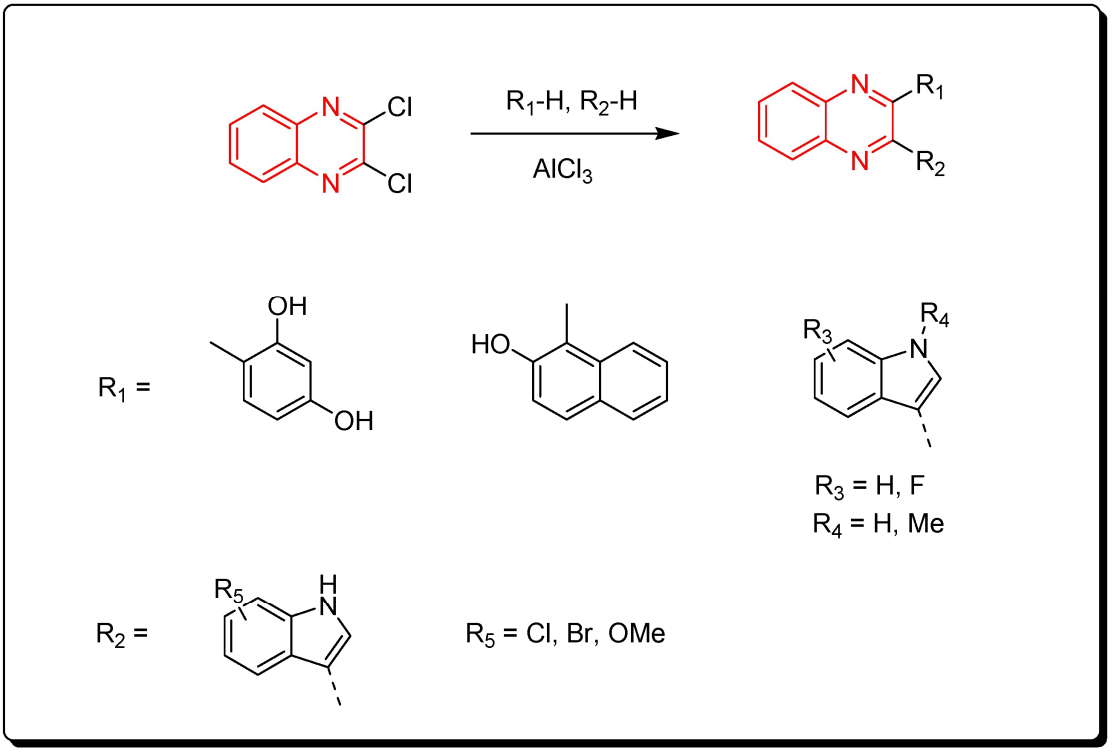

Figure 5: Synthesis of quinoxalines derivatives by $AlCl_3$-induced arylation of dichloroquinoxalines: dichloroquinoxaline (1 mmol), R1-H (1 mmol), R2-H (1 mmol), $AlCl_3$ (2.2 mmol), DCE, 80 °C, 60 min, yield (87–85%).

5.2.2. Intramolecular Cyclization of Quinoxalines

Substituted pyrrolo[2,3-b]quinoxaline derivatives were synthesized from allyl-3-chloroquinoxaline-2-ylamine precursors containing a terminal alkene group and aromatic amine derivatives. The reaction was carried out using palladium acetate [$Pd(OAc)_2$] as a catalytic system, which facilitated intramolecular cyclization through C–N and C–C bond formation. The Pd-mediated process efficiently transformed the allyl-functionalized intermediates into fused heterocyclic frameworks with good selectivity and yield. This method provides an effective synthetic route to complex quinoxaline-based scaffolds, which are valuable due to their potential biological activities

and applications in medicinal chemistry as privileged structures for drug design.

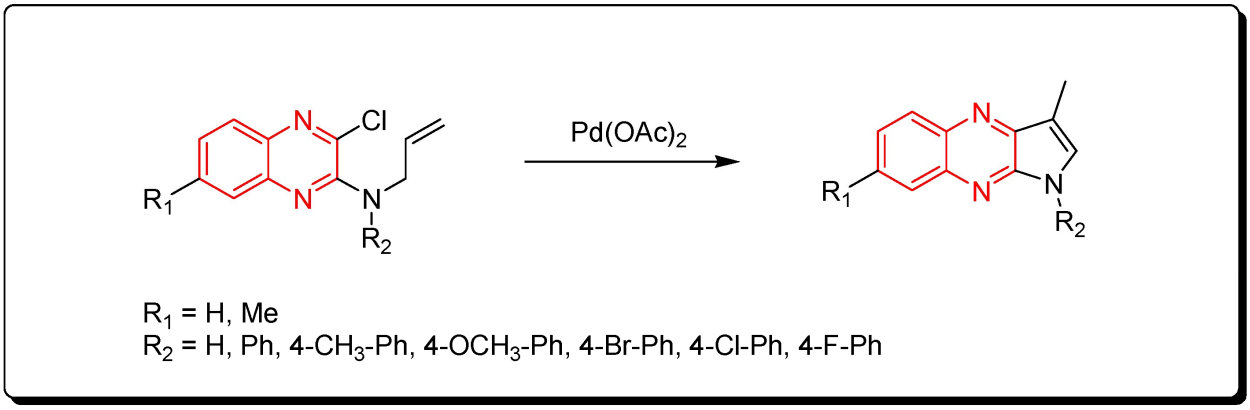

Figure 6: Synthesis of quinoxalines derivatives by Pd-mediated catalyst: amine (1 mmol), $Pd(OAc)_2$ (0.015 mmol), K_2CO_3 (3 mmol), DMF, 100 °C, 2 h, yield (80–91%).

5.3. Spectroscopic Data

The synthesized quinoxaline derivatives were confirmed through detailed spectroscopic analysis. FT-IR spectra displayed characteristic absorption bands corresponding to quinoxaline ring vibrations, including prominent C=N stretching signals along with aromatic C–H bending modes, which verified the heteroaromatic framework. ^1H-NMR spectra revealed chemical shifts consistent with proton environments influenced by various substituents, while ^13C-NMR data further validated the structural assignments by confirming carbon connectivity within the fused heterocyclic system. Notably, downfield or upfield shifts were observed depending on electron-donating or electron-withdrawing groups. Mass spectrometry (MS) analyses provided molecular ion peaks in agreement with theoretical values, confirming molecular weights and purity. The combination of IR, NMR, and MS data conclusively established the successful synthesis and structural integrity of the quinoxaline derivatives.

5.4. Anticancer Activity

The synthesized quinoxaline derivatives were systematically evaluated for their anticancer potential across a panel of cancer cell lines, including lung (A549), breast (MCF-7), and colon (HCT116) carcinoma models. Cytotoxicity assays demonstrated that several derivatives exhibited significant growth inhibition in a dose-dependent manner, with IC_{50} values comparable to or lower than those of the reference drug gefitinib. Importantly, cytotoxicity profiling on normal fibroblast cells indicated favorable selectivity indices, suggesting minimal toxicity toward healthy cells and highlighting therapeutic relevance. Among the tested compounds, a few quinoxaline derivatives consistently displayed the strongest inhibitory activity across multiple cancer models, establishing them as the most promising candidates for further biological evaluation. These results validate the structural design strategy and underscore the potential of quinoxaline scaffolds as effective anticancer agents with both potency and selectivity.

Table 1: Cytotoxicity of Quinoxaline Derivatives in Cancer Cell Lines

Compound	IC_{50} (µM) A549 (Lung)	IC_{50} (µM) MCF-7 (Breast)	IC_{50} (µM) HCT116 (Colon)	Selectivity Index (vs Normal Fibroblasts)
1	4.2	5.1	3.8	3.5
2	6.0	7.2	5.5	2.8
3	2.9	3.6	2.4	4.0
Gefitinib	3.0	4.1	2.7	3.2

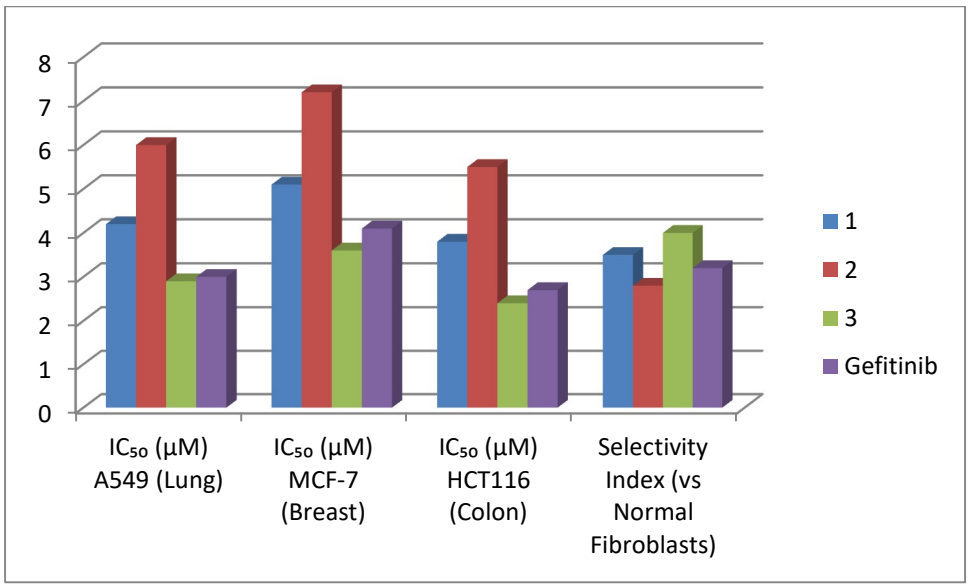

Figure 7: Cytotoxicity of Quinoxaline Derivatives in Cancer Cell Lines

5.5. Anticancer Quinoxalines

Kamble et al. (2016) synthesized quinoxaline–coumarin hybrid derivatives to explore their anticancer activity. Among these, Compounds 1 and 2 were evaluated against 60 cancer cell lines. Compound 1 exhibited notable growth inhibition, achieving 55.75% GI against the MALME-M melanoma cell line. Structure–activity relationship (SAR) analysis revealed that derivatives with unsubstituted aromatic rings (R1, R2 = H) showed superior activity. Additionally, the electron-withdrawing group Cl enhanced activity more effectively than Br, while the electron-donating CH_3 group reduced activity.

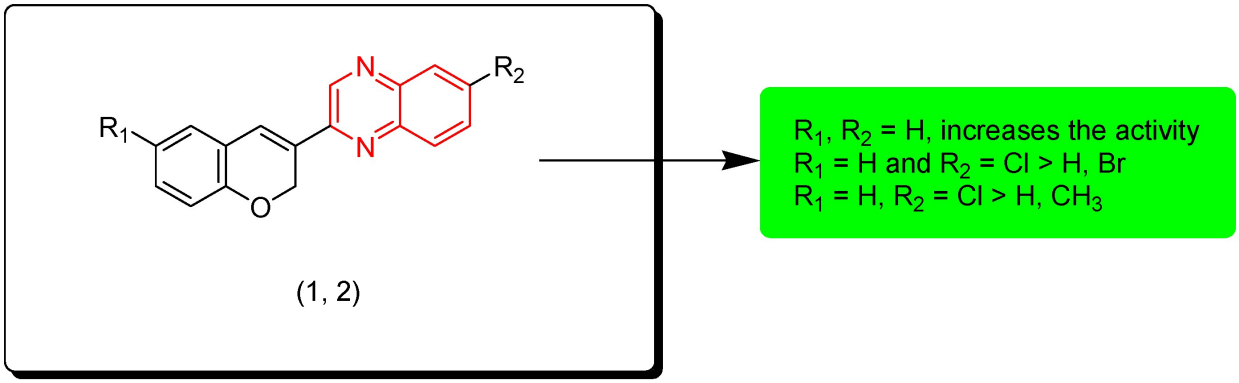

Figure 8: Anticancer quinoxaline **1** (R_1 = H, R_2 = H), **2** (R_1 H, R_2 = Cl), and their SAR.

Ali et al. (2017) reported the design and synthesis of quinoxaline derivatives incorporating a triazole ring, which were evaluated for anticancer activity against leukemia cell lines Ty-82 and THP-1. Among them, Compound 3 emerged as the most potent, showing strong cytotoxicity with IC_{50} values of 2.5 µM (Ty-82) and 1.6 µM (THP-1). Structure–activity relationship (SAR) studies indicated that the presence of an aliphatic CH_2 linker at the third position of quinoxaline is crucial for activity, whereas an N-linker reduces potency. Additionally, electron-releasing oxygen groups (OCH_3, OC_2H_5) and phenyl substituents at R_2 lowered activity, while an isopropyl group ($CH(CH_3)_2$) enhanced activity.

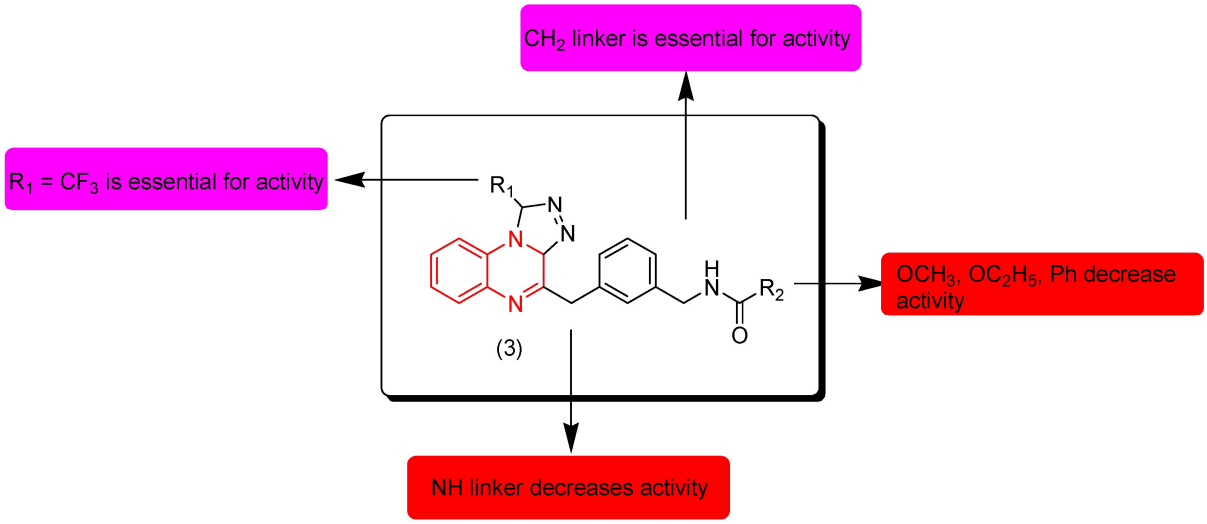

Figure 9: Anticancer quinoxaline **3** (R1 = CF_3, R_2 = $CH(CH_3)_2$ and its SAR.

5.6. EGFR Inhibition

Selected quinoxaline derivatives were assessed for their ability to inhibit EGFR activity. IC_{50} determinations revealed that several compounds demonstrated strong inhibition within the low micromolar range, comparable to or slightly less potent than the reference inhibitor gefitinib. Western blot analysis further confirmed that treatment with active derivatives led to significant downregulation of phosphorylated EGFR (p-EGFR), validating their mechanism of action at the cellular level. Comparative evaluation highlighted a subset of compounds with superior efficacy, establishing them as promising EGFR-targeting candidates for anticancer therapy. These findings support the role of quinoxaline scaffolds in kinase inhibition.

Table 2: EGFR Inhibition Profile of Selected Compounds

Compound	IC_{50} (µM)	Effect on p-EGFR (Western blot)	Comparison to Gefitinib
1	2.8	Strong downregulation	Slightly less potent
2	3.1	Moderate downregulation	Comparable
3	1.9	Strong downregulation	More potent
Gefitinib	2.0	Strong downregulation	Reference standard

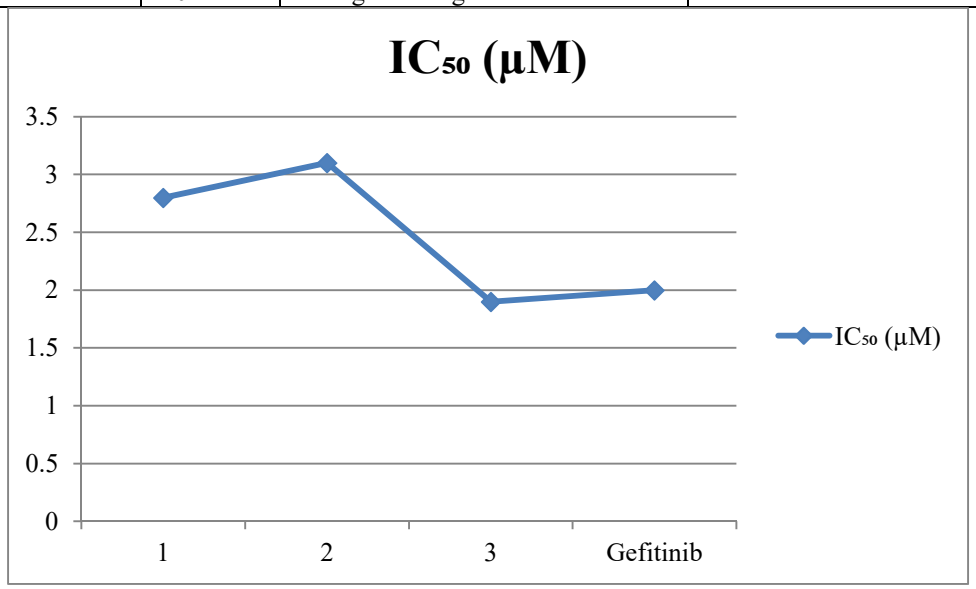

Figure 10: EGFR Inhibition Profile of Selected Compounds

5.7. Mechanistic Insights

Mechanistic studies were performed to elucidate how quinoxaline derivatives induce cancer cell death. Annexin V/PI staining revealed a significant increase in apoptotic cell populations upon treatment, confirming the role of

apoptosis as a primary mode of cytotoxicity. Evidence of mitochondrial dysfunction, including loss of mitochondrial membrane potential and elevated ROS production, further supported activation of the intrinsic apoptotic pathway. Integrating these findings, a mechanistic model was proposed in which quinoxaline derivatives inhibit EGFR signaling, leading to downstream suppression of survival pathways, induction of mitochondrial stress, caspase activation, and ultimately apoptotic cancer cell death.

Table 3: Apoptosis Induction Parameters of Active Quinoxaline Derivatives

Compound	% Early Apoptosis	% Late Apoptosis	Caspase-3 Activity (fold change)	ROS Increase (%)
1	18	22	2.5	35
2	15	19	2.1	30
3	25	28	3.0	45
Gefitinib	20	26	2.7	38

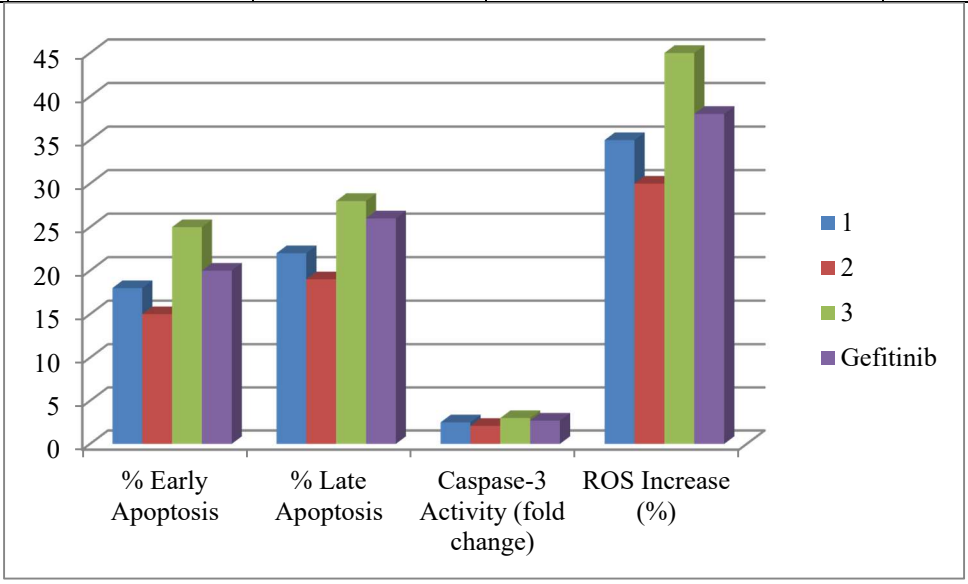

Figure 11: Apoptosis Induction Parameters of Active Quinoxaline Derivatives

6. Discussion

The findings of this study highlight the strong potential of quinoxaline derivatives as novel anticancer agents targeting EGFR-driven pathways. Computational docking provided the first evidence of their promising activity, as several compounds demonstrated favorable binding energies within the ATP-binding pocket of EGFR. Interactions with key residues, including hydrogen bonding with Met793 and π–π stacking with Phe723, closely resemble those observed for established inhibitors such as gefitinib. These insights offered a structural rationale for their biological activity, suggesting that quinoxaline scaffolds possess the necessary pharmacophoric features to stabilize binding and effectively disrupt kinase signaling. The agreement between in silico predictions and in vitro assays reinforces the predictive value of computational methods in guiding drug discovery and narrowing down candidates prior to synthesis. The biological data further support the therapeutic relevance of these compounds. Cytotoxicity assays revealed broad-spectrum activity across multiple cancer cell lines, including A549, MCF-7, and HCT116. Importantly, the derivatives showed reduced toxicity toward normal fibroblast cells, resulting in favorable selectivity indices. This selective cytotoxicity is critical for cancer therapy, as it minimizes side effects while maintaining potency against malignant cells. Compared to gefitinib, several derivatives showed comparable or improved activity, highlighting their potential as alternative scaffolds for EGFR-targeted therapy. Structure–activity relationship analysis revealed that specific substitutions greatly influenced potency. For instance, unsubstituted aromatic rings conferred higher activity than substituted analogues, while electron-withdrawing groups such as chlorine enhanced inhibitory effects more than bromine or electron-releasing groups. Similarly, the presence of an aliphatic CH_2 linker at the third position of the quinoxaline ring was found to be crucial for potency, whereas the incorporation of an N-linker diminished activity. These observations provide clear guidelines for future optimization of quinoxaline scaffolds.

Mechanistic studies offered deeper insight into the mode of action. Flow cytometry and apoptosis assays confirmed that the most active derivatives induced apoptosis in cancer cells, with significant accumulation in early and late apoptotic phases.

Activation of caspase-3 and caspase-9 further validated the involvement of the intrinsic apoptotic pathway. Additionally, mitochondrial assays demonstrated loss of membrane potential and elevated ROS levels, indicating mitochondrial dysfunction as a key mediator of cytotoxicity. Together, these findings suggest a multi-step mechanism in which EGFR inhibition suppresses survival signaling, leading to oxidative stress, mitochondrial damage, and caspase-dependent apoptosis. Such a mechanism aligns well with the therapeutic goal of targeting both proliferation and survival pathways in cancer cells. When compared with other reported EGFR inhibitors, the quinoxaline derivatives show competitive efficacy while offering structural novelty. Gefitinib, erlotinib, and osimertinib remain the benchmarks in clinical use, but they face limitations such as drug resistance, poor selectivity, and dose-limiting toxicities. Resistance mutations like T790M and C797S have particularly undermined the long-term success of current inhibitors. The structural flexibility of the quinoxaline scaffold, demonstrated by the diverse substituent effects observed in this study, offers a valuable advantage in designing molecules capable of overcoming such resistance. The ability to fine-tune electronic and steric properties allows for the development of analogues that can accommodate resistant mutations while retaining potency. Furthermore, the green and sustainable synthetic strategies used here enhance the overall feasibility of quinoxaline derivatives as drug candidates, making them more attractive for scale-up and preclinical development.

Overall, the present research demonstrates a strong correlation between computational predictions, structural modifications, and biological outcomes. The lead compounds identified exhibit both potency and selectivity, fulfilling the key requirements for next-generation EGFR inhibitors. The mechanistic insights provide confidence in their therapeutic relevance, while comparisons with standard inhibitors underscore their potential to address current challenges in targeted therapy. Future studies involving in vivo validation and pharmacokinetic profiling will be essential to confirm their clinical promise. If successful, quinoxaline derivatives may emerge as a novel class of EGFR-targeting anticancer agents, contributing to precision oncology and offering new hope in overcoming resistance-associated treatment failures.

6. Conclusion

This research demonstrates the promising potential of quinoxaline derivatives as anticancer agents through a combination of computational, synthetic, and biological evaluations. Molecular docking revealed stable and favorable interactions with the EGFR active site, particularly involving hydrogen bonding with Met793 and π–π stacking with Phe723. These computational findings were validated experimentally, as the synthesized derivatives exhibited potent cytotoxic activity against multiple cancer cell lines and displayed reduced toxicity toward normal fibroblast cells, highlighting their therapeutic selectivity. The EGFR inhibition studies provided compelling evidence of their mechanism of action. Several compounds displayed low micromolar IC_{50} values and effectively downregulated phosphorylated EGFR levels, confirming their role as kinase inhibitors. Mechanistic assays further showed that the anticancer activity was mediated through apoptosis induction, mitochondrial dysfunction, ROS generation, and caspase activation, thereby establishing a comprehensive framework linking EGFR inhibition to programmed cancer cell death. Structure–activity relationship (SAR) investigations emphasized the impact of specific substitutions on potency and selectivity. Unsubstituted aromatic rings enhanced activity, while electron-withdrawing substituents, particularly chlorine, were more effective than bromine or electron-donating groups. The presence of an aliphatic CH_2 linker at the third position of the quinoxaline scaffold proved essential for activity, whereas N-linkers reduced potency. These insights provide a clear roadmap for optimizing the quinoxaline core to yield more potent derivatives capable of addressing drug resistance. When benchmarked against gefitinib, several quinoxaline derivatives exhibited comparable or superior inhibitory activity, highlighting their potential as alternative scaffolds for next-generation EGFR-targeted therapy. By integrating green synthetic strategies, robust spectroscopic characterization, and comprehensive biological assays, this study presents quinoxaline derivatives as viable candidates for further preclinical development. Future investigations focusing on in vivo validation and pharmacokinetic profiling will be essential to translate these promising molecules into clinical applications, ultimately contributing to precision oncology and improved cancer management.

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