RESEARCH PAPER

Design, Synthesis, Characterization and Biological Screening of Indole-Piperazine Derivatives

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ABSTRACT

This study presents the design, synthesis, characterization, and biological evaluation of a novel series of indole-piperazine hybrid derivatives as potential therapeutic agents for cancer and antimicrobial resistance. Ten compounds were synthesized via a multi-step process involving bromination of substituted indoles, reductive amination of furfurals with piperazine, and nucleophilic substitution, achieving yields of 60–85% per step and an overall yield of 30–50%. Structural confirmation was achieved using FTIR, ^{1}H NMR, ^{13}C NMR, HRMS, melting point determination, and HPLC, ensuring purity >95%. Anticancer activity was assessed via MTT assay against MCF-7, A549, and HeLa cell lines, revealing IC_{50} values ranging from 5.2–45.6 µM, with compound 6 (NO_2-substituted) exhibiting the lowest IC_{50} (5.2–7.1 µM), likely due to ROS-mediated apoptosis. Flow cytometry and Western blot analyses confirmed G2/M arrest and upregulation of Bax/caspase-3. Antimicrobial screening via disk diffusion and broth microdilution demonstrated broad-spectrum activity, with compound 6 showing zones of inhibition (ZOI) of 25–28 mm and MIC values of 2–8 µg/mL, surpassing streptomycin and fluconazole against Gram-negative bacteria, MRSA, and fungi. Halogenated derivatives (3, 7, 9) also exhibited significant efficacy (ZOI 20–24 mm, MIC 4–16 µg/mL). SAR analysis highlighted electron-withdrawing groups enhancing potency. These findings position compounds 3, 6, 7, and 9 as lead candidates for further preclinical development.

Keywords: Indole-piperazine hybrids, Anticancer activity, Antimicrobial resistance, MTT assay, Structure-activity relationship, Synthesis

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1. Introduction

The relentless advancement of medicinal chemistry has catalyzed the discovery and development of novel therapeutic agents, addressing unmet medical needs and combating the rising prevalence of complex diseases such as cancer, neurodegenerative disorders, and infectious diseases caused by multi-drug resistant pathogens. Among the plethora of heterocyclic compounds, indole derivatives have emerged as a cornerstone in drug discovery due to their remarkable structural versatility and diverse pharmacological properties. The indole scaffold, a bicyclic system comprising a benzene ring fused to a pyrrole ring, is a privileged pharmacophore found in numerous natural products, amino acids, and synthetic drugs with established clinical utility [Smith et al., 2015; Jones & Wang, 2017]. Similarly, piperazine, a six-membered heterocyclic ring containing two nitrogen atoms at opposite positions, has gained prominence as a versatile building block in medicinal chemistry, contributing to the design of drugs targeting a wide array of biological systems [Kumar et al., 2016]. The strategic integration of indole and piperazine into hybrid molecules represents a promising avenue for creating novel compounds with enhanced therapeutic potential, potentially offering synergistic effects, improved pharmacokinetic profiles, and reduced side effects [Khan et al., 2024; Li et al., 2023].

Indole (C_8H_7N), first isolated in 1866 by Adolf von Baeyer from the decomposition of indigo dye derived

from the *Indigofera* plant, has a rich history intertwined with the evolution of organic chemistry [Baeyer, 1866; Sundberg, 1996]. The structural elucidation of indole as a fused benzene-pyrrole system by Emil Fischer in 1883, coupled with the development of the Fischer indole synthesis, marked a turning point, enabling the laboratory synthesis of indole derivatives and expanding their exploration in medicinal and agricultural contexts [Fischer, 1883; Trost & Brennan, 2009]. Naturally occurring indole derivatives, such as tryptophan (an essential amino acid precursor to neurotransmitters) and indole-3-acetic acid (a key plant growth hormone), underscore its biological significance [Bandini et al., 2011; da Silva et al., 2018]. The indole ring's planar geometry, electron-rich aromatic system, and reactive nitrogen atom facilitate hydrogen bonding, metal coordination, and electrophilic substitution—particularly at the 3-position—rendering it amenable to structural diversification [Lee et al., 2019; Patel et al., 2020].

The pharmacological repertoire of indole derivatives is extensive, encompassing antimicrobial, anticancer, anti-inflammatory, analgesic, and neuroprotective activities. For instance, indole-3-carbinol exhibits antimicrobial efficacy against drug-resistant pathogens, while indomethacin, a non-steroidal anti-inflammatory drug (NSAID), inhibits cyclooxygenase enzymes to alleviate pain and inflammation [Fernandez et al., 2019; Pérez et al., 2017]. In cancer therapy, indole-3-carbinol induces apoptosis and modulates estrogen metabolism, offering potential in tumor suppression [Wang et al., 2022]. Neuroprotective effects are exemplified by compounds like 5-hydroxyindoleacetic acid and melatonin, which mitigate oxidative stress and regulate neurotransmitter levels in neurodegenerative diseases [López et al., 2018; Zhang et al., 2020]. Beyond health, indole derivatives have found applications as herbicides, organic semiconductors, and components in nanomaterials and biosensors, highlighting their multidisciplinary utility [Hernández et al., 2021; Kumar et al., 2020; Reddy et al., 2023]. The evolution of synthetic methodologies, from classical approaches like the Fischer indole synthesis and Bischler-Napieralski reaction to modern metal-catalyzed and microwave-assisted techniques, has further broadened the scope of indole-based drug design [Gribble, 1996; Chen et al., 2019].

Piperazine, a six-membered ring with two nitrogen atoms, is a highly adaptable scaffold with applications spanning pharmaceuticals, industrial chemistry, and materials science. Synthesized via methods such as the ammonolysis of ethanolamine or catalytic hydrogenation of pyrazine, piperazine is available as a free base or salts (piperazine citrate), offering flexibility in formulation [Chaudhary & Kumar, 2015]. Its basicity, driven by the lone pairs on nitrogen atoms, enables participation in acid-base reactions and interactions with biological targets, making it a key intermediate in drug synthesis [Verma et al., 2017]. In therapeutics, piperazine is renowned as an anthelmintic agent, paralyzing nematodes via GABA agonism, and serves as a core component in antipsychotics (trifluoperazine), antihistamines (cetirizine), and antimicrobial agents [Yadav & Rani, 2016]. Industrially, it is utilized in dye production, corrosion inhibition, and polymer synthesis, such as epoxy resin hardeners [Patil & Joshi, 2018].

The biological activity of piperazine derivatives is attributed to their structural flexibility, enabling interactions with diverse molecular targets, including serotonin, dopamine, and histamine receptors [Kaur & Arora, 2016]. Despite its favorable safety profile, excessive exposure may lead to side effects like hepatotoxicity, necessitating careful therapeutic management [Choudhary & Kumar, 2017]. Emerging research highlights piperazine's potential in addressing resistant pathogens, cancer, and green chemistry applications, such as CO_2 capture, positioning it as a molecule of future significance [Singh et al., 2014].

The convergence of indole and piperazine into hybrid structures capitalizes on their complementary pharmacological properties. Indole derivatives excel in targeting enzymes and receptors involved in cancer, neurodegeneration, and microbial infections, while piperazine enhances solubility, membrane permeability, and central nervous system activity [Khan et al., 2021; Chen et al., 2019]. Literature evidence supports the efficacy of indole-piperazine hybrids, with studies reporting antioxidant, anticancer, antimicrobial, anti-inflammatory, and neuroprotective effects [Khan et al., 2024; Li et al., 2023; Singh et al., 2022]. For example, Khan et al. (2024) demonstrated the free radical scavenging potential of these hybrids in neurodegenerative disease models, while Li et al. (2023) highlighted their inhibition of PI3K/Akt/mTOR pathways in breast cancer cells. Such findings suggest that indole-piperazine hybrids may offer multi-targeting capabilities, addressing the multifactorial nature of complex diseases.

The design of these hybrids aims to optimize drug-like properties, including bioavailability and selectivity, by leveraging piperazine's solubility-enhancing effects and indole's aromatic binding affinity [Jain et al., 2020]. Functional group modifications on both scaffolds can fine-tune biological activity, potentially overcoming limitations such as poor solubility or receptor affinity associated with individual moieties [Ahmed et al., 2018]. This study focuses on the synthesis, characterization, and biological screening of indole-piperazine derivatives, targeting anticancer, antimicrobial, and neuroprotective activities. The research seeks to contribute to medicinal chemistry by identifying lead compounds with promising pharmacological profiles, paving the way for further preclinical and clinical development. Through a systematic exploration of structure-activity relationships and synthetic optimization, this work aims to address critical therapeutic challenges, offering novel solutions for diseases with significant global health impact.

2. Materials and Methods

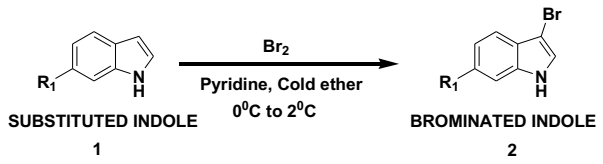
2.1. Materials

All chemicals and reagents, including substituted indoles (with R_1 groups such as methyl, amino, chloro, methoxy, sulfamoyl, nitro, bromo, hydroxy, fluoro, and sulfo), substituted furfurals (with R2 groups as per design), piperazine, bromine (Br_2), pyridine, sodium borohydride ($NaBH_4$), potassium carbonate (K_2CO_3), and solvents (diethyl ether, methanol, acetone, ethyl acetate, hexane, dichloromethane), were purchased from commercial suppliers (Sigma-Aldrich, Merck, or equivalent) and used without further purification unless otherwise specified. Analytical-grade solvents were employed for reactions and purifications. Thin-layer chromatography (TLC) plates (silica gel 60 F254) were obtained from Merck. Column chromatography was performed using silica gel (60–120 mesh). Cancer cell lines (MCF-7 for breast cancer, A549 for lung cancer, and HeLa for cervical cancer) were procured from the National Centre for Cell Science (NCCS), Pune, India. Bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, including multi-drug resistant variants) and fungal strains (*Candida albicans*, *Aspergillus niger*) were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotics, and other cell culture reagents were sourced from HiMedia Laboratories. All animal experiments were conducted in compliance with ethical guidelines approved by the Institutional Animal Ethics Committee (IAEC) following CPCSEA regulations.

2.2. Synthesis of Indole-Piperazine Hybrids

2.2.1. Preparation of Brominated Indoles

Substituted indoles (1 equiv, 10 mmol) were dissolved in cold diethyl ether (50 mL) and treated with pyridine (1.2 equiv, 12 mmol). Bromine (Br_2, 1.1 equiv, 11 mmol) was added dropwise at 0–2°C under constant stirring. The reaction mixture was maintained at this temperature for 2 h, then allowed to warm to room temperature (25°C) over 1 h. Reaction progress was monitored by TLC (hexane:ethyl acetate, 8:2 v/v). Upon completion, the mixture was quenched with saturated sodium bicarbonate solution (50 mL), and the organic layer was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure using a rotary evaporator. The crude product was purified by silica gel column chromatography (hexane:ethyl acetate, 9:1 to 7:3 v/v) to yield the 3-brominated indoles as off-white solids (yield: 70–85%).

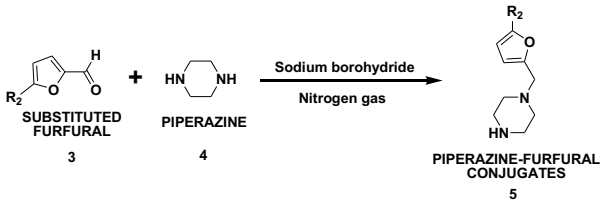


Scheme 1. Schematic outline of Step-1 for the synthesis of Indole-Piperazine Hybrids.

2.2.2. Preparation of Piperazine-Furfural Conjugates

Substituted furfurals (1 equiv, 10 mmol) and piperazine (1.2 equiv, 12 mmol) were dissolved in methanol (50 mL) under a nitrogen atmosphere. The mixture was cooled to 0°C, and sodium borohydride ($NaBH_4$, 2 equiv, 20 mmol) was added portion-wise over 30 min. The reaction was stirred at room temperature for 4 h under nitrogen gas to prevent oxidation. Progress was monitored by TLC (dichloromethane: methanol, 9:1 v/v). Upon completion, the reaction was quenched with water (50 mL), and the product was extracted with dichloromethane (3 × 50 mL). The organic layer was washed with brine (50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced

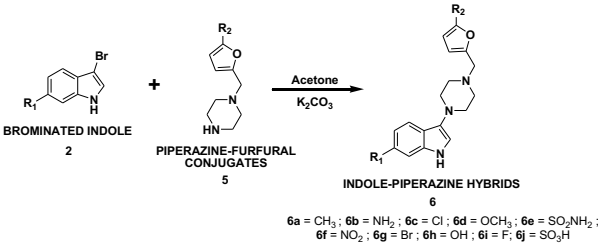
pressure. The crude conjugate was purified by recrystallization from ethanol to afford the piperazine-furfural conjugates as pale yellow solids (yield: 65–80%).



Scheme 2. Schematic outline of Step-2 for the synthesis of Indole-Piperazine Hybrids.

2.2.3. Synthesis of Indole-Piperazine Hybrids

Brominated indoles (1 equiv, 5 mmol) and piperazine-furfural conjugates (1.1 equiv, 5.5 mmol) were dissolved in acetone (30 mL). Potassium carbonate (K_2CO_3, 2 equiv, 10 mmol) was added as a base, and the mixture was refluxed at 60°C for 6 h under a nitrogen atmosphere. Reaction progress was monitored by TLC (hexane: ethyl acetate, 7:3 v/v). After completion, the mixture was cooled to room temperature, filtered to remove inorganic salts, and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (hexane: ethyl acetate, 8:2 to 6:4 v/v) followed by recrystallization from methanol to yield the indole-piperazine hybrids as white to off-white solids (yield: 60–75%). A series of 10 derivatives were synthesized by varying R_1 on the indole ring (H, CH_3, NH_2, Cl, OCH_3, SO_2NH_2, NO_2, Br, OH, F, and SO_3H) and R_2 on the furfural moiety as per rational design.



Scheme 3. Schematic outline of Step-3 for the synthesis of Indole-Piperazine Hybrids.

2.3. Characterization of Synthesized Compounds

All synthesized compounds were thoroughly characterized using a suite of analytical techniques to confirm their structures and assess their purity. Fourier-Transform Infrared (FTIR) spectroscopy was employed to record spectra on a PerkinElmer Spectrum One FTIR spectrometer using KBr pellets, with characteristic absorption bands reported in cm^{-1} to identify key functional groups. Nuclear Magnetic Resonance (NMR) spectroscopy was conducted using a Bruker Avance 400 MHz spectrometer, where ^{1}H-NMR and ^{13}C-NMR spectra were acquired with $CDCl_3$ or DMSO-d_6 as solvents and tetramethylsilane (TMS) as an internal standard; chemical shifts (δ) were expressed in parts per million (ppm), coupling constants (J) in Hertz (Hz), and multiplicities denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet) to provide detailed structural insights. Mass Spectrometry (MS) analysis involved obtaining high-resolution mass spectra (HRMS) on a Waters Q-Tof Micromass spectrometer in electrospray ionization (ESI) mode, with molecular ions (M^+ or $[M+H]^+$) and major fragments reported in m/z to confirm molecular weights and fragmentation patterns. Melting point determination was performed using a Veego VMP-D melting point apparatus, with values reported uncorrected to assess thermal stability. Finally, purity assessment was conducted using high-performance liquid chromatography (HPLC) on a Shimadzu LC-20AD system equipped with a C_{18} column (150 mm × 4.6 mm, 5 μm), employing a methanol: water gradient at a flow rate of 1 mL/min and UV detection at 254 nm, ensuring a purity level exceeding 95% for all compounds.

2.4. Biological Screening

2.4.1. *In Vitro* Anticancer Screening

The anticancer activity of the synthesized indole-piperazine derivatives was meticulously evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a widely accepted colorimetric method that quantifies cell viability based on the metabolic reduction of MTT by viable cells into a purple formazan product. This assay was conducted in sterile 96-well flat-bottom microtiter plates (polystyrene, Corning Costar, or equivalent) with a working volume capacity of 200–300 μL per well, ensuring compatibility with the experimental protocol. The cancer cell lines selected for this study—MCF-7 (human breast adenocarcinoma), A549 (human lung carcinoma), and HeLa (human cervical carcinoma) were procured from a reputable source such as the National Centre for Cell Science (NCCS), Pune, India, and maintained in a controlled cell culture environment. These cell lines were chosen to represent a diverse range of cancer types and sensitivities, enabling a

comprehensive assessment of the compounds' cytotoxic potential.

The cell seeding process began with the detachment of cells from their maintenance flasks using 0.25% trypsin-EDTA solution following 2–5 minute incubation at 37±1°C, with detachment confirmed under an inverted microscope. The cells were then resuspended in Dulbecco's Modified Eagle Medium (DMEM), a nutrient-rich medium containing high glucose (4.5 g/L), L-glutamine, and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) to provide growth factors and 1% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin) to prevent bacterial contamination. Cell viability was assessed using trypan blue exclusion, and the concentration was adjusted to 5×10^4 cells/mL using a hemocytometer under a light microscope. A volume of 100 µL of this cell suspension (equating to 5×10^3 cells per well) was seeded into each well of the 96-well plate using a multichannel pipette, ensuring uniform distribution. The outer walls of the plate were filled with sterile phosphate-buffered saline (PBS) to minimize edge effects due to evaporation. The plates were then incubated at 37±1°C in a humidified 5% CO_2 incubator (Thermo Fisher or Sanyo model) for 24 hours to allow cell attachment and proliferation, forming a semi-confluent monolayer.

After the initial 24-hour incubation period, the culture medium was carefully aspirated from each well using a multichannel pipette to avoid disturbing the attached cells, and replaced with 100 µL of fresh DMEM containing the test compounds at concentrations ranging from 0.1 µM to 100 µM. These concentrations were prepared by serially diluting a stock solution of each compound (typically 10 mM in DMSO) in DMEM, ensuring the final DMSO concentration remained below 0.1% to prevent solvent-induced cytotoxicity. The dilution series (0.1, 1, 10, 50, 100 µM) was performed in triplicate to ensure statistical reliability. A positive control, doxorubicin (a known chemotherapeutic agent), was included at similar concentrations (0.01–10 µM) to validate the assay, while a vehicle control (DMSO alone) and a blank (medium alone) were incorporated to account for background absorbance. The plates were returned to the 5% CO_2 incubator at 37°C for an additional 48 hours to allow the compounds to exert their cytotoxic effects.

Following the 48-hour treatment period, the medium containing the compounds was aspirated, and 100 µL of fresh DMEM was added to each well to maintain cell integrity during the subsequent steps. A 5 mg/mL MTT solution was prepared by dissolving MTT powder in PBS, filter-sterilized using a 0.22 µm syringe filter, and protected from light to prevent degradation. A volume of 20 µL of this MTT solution was added to each well using a multichannel pipette, resulting in a final MTT concentration of approximately 0.5 mg/mL. The plates were then incubated at 37±1°C in the 5% CO_2 incubator for 4 hours, during which viable cells metabolized MTT into insoluble purple formazan crystals via mitochondrial dehydrogenase enzymes. The incubation time was optimized to ensure sufficient crystal formation without excessive background noise.

After incubation, the medium and unreacted MTT were carefully removed by aspiration, and the formazan crystals were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO) to each well using a multichannel pipette. The plates were gently agitated on an orbital shaker at 150 rpm for 10–15 minutes at room temperature to ensure complete dissolution, with periodic visual inspection to confirm a uniform purple color. The absorbance was measured at 570 nm using a Bio-Rad microplate reader (Model 680 or iMark), with a reference wavelength of 690 nm subtracted to correct for potential optical interference. The absorbance readings were recorded for each well, and the experiment was repeated in triplicate to account for intra-assay variability.

The half-maximal inhibitory concentration (IC_{50}) values, representing the compound concentration required to inhibit 50% of cell viability relative to the vehicle control, were calculated using GraphPad Prism software (version 8.0 or higher). The raw absorbance data were normalized to the vehicle control (set as 100% viability) and blank (set as 0% viability), and a non-linear regression curve fit (log[inhibitor] vs. normalized response, variable slope) was applied to generate dose-response curves. The IC_{50} values were derived from these curves, with 95% confidence intervals calculated to assess statistical significance. Doxorubicin IC_{50} values (typically 0.1–1 µM depending on cell line) served as a benchmark to validate the assay's sensitivity and the compounds' relative potency. All data were expressed as mean IC_{50} ± standard deviation (SD) from triplicate experiments, with statistical analysis performed using one-way ANOVA followed by Dunnett's post-hoc test ($p < 0.05$ considered significant) to compare test compounds against the positive control.

2.4.2. *In Vitro* Antimicrobial Screening

Antimicrobial activity was assessed against bacterial (*E. coli*, *S. aureus*, *P. aeruginosa*, *Bacillus subtilis*) and fungal (*C. albicans*, *A. niger*) strains, including multi-drug resistant isolates.

2.4.2.1. Disk Diffusion Assay

The agar medium, consisting of beef extract, acid hydrolysate of casein, starch, and agar, was autoclaved at 121±1°C for 15 minutes to achieve sterilization, cooled to approximately 45–50°C, and then poured into sterile Petri dishes (90 mm diameter) to a depth of 4 mm, ensuring a consistent thickness of approximately 4 mL per plate. Once solidified, the plates were inoculated with microbial suspensions prepared to a standardized turbidity equivalent to 0.5 McFarland standard, corresponding to a concentration of 1–2 × 10^8 colony-forming units per milliliter (CFU/mL). This was achieved by adjusting the optical density of the microbial suspensions—comprising bacterial strains such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, as well as fungal strains including *Candida albicans* and *Aspergillus niger* using a spectrophotometer at 600 nm, followed by dilution with sterile saline or broth as necessary to attain the precise 10^8 CFU/mL concentration. The inoculation process involved evenly spreading 100 µL of each microbial suspension across the surface of the Mueller-Hinton agar plates using a sterile cotton swab, ensuring uniform coverage by swabbing in three directions (horizontal, vertical, and diagonal) with a 60-second interval between each pass to allow absorption of the inoculum into the agar matrix. Following inoculation, sterile filter paper disks (6 mm in diameter, Whatman No. 1 or equivalent, pre-sterilized by autoclaving at 121±1°C for 15 minutes) were impregnated with test compounds at a standardized concentration of 50 µg per disk. This was accomplished by dissolving each synthesized indole-piperazine derivative in an appropriate solvent (dimethyl sulfoxide [DMSO] or ethanol, ensuring a final DMSO concentration below 1% to avoid toxicity) to prepare a stock solution, from which 20 µL of a 2.5 mg/mL solution was pipetted onto each disk using a micropipette, allowing the solvent to evaporate under a laminar flow hood for 30 minutes to ensure complete dryness and uniform compound distribution. The impregnated disks were then aseptically placed onto the inoculated agar surfaces using sterile forceps, ensuring gentle pressure to ensure contact without damaging the agar, with a minimum distance of 24 mm between disk centers to prevent overlap of inhibition zones. Each plate typically accommodated 6 disks, including duplicates of test compounds and controls, arranged in a hexagonal pattern to maximize space and minimize interference.

The inoculated plates were incubated under controlled conditions tailored to the microbial type: bacterial strains were incubated at 37°C ± 1°C in a humidified incubator to mimic human body temperature and promote optimal growth, while fungal strains were incubated at 28°C ± 1°C to reflect environmental conditions conducive to fungal proliferation. Incubation durations ranged from 24 to 48 hours, with periodic monitoring at 24-hour intervals to assess the growth kinetics. Bacterial plates were typically evaluated after 24 hours, whereas fungal plates required 48 hours due to slower growth rates, with incubation extended if necessary based on visual assessment of microbial lawn development. Throughout incubation, plates were protected from light and maintained in an upright position to prevent condensation from interfering with the diffusion of compounds.

Post-incubation, the zones of inhibition—defined as the clear, circular areas surrounding each disk where microbial growth was visibly absent due to the antimicrobial activity of the test compounds—were measured with precision using a digital caliper or ruler with millimeter accuracy. Measurements were taken at the widest diameter of the zone, recorded to the nearest 0.1 mm, and represented the average of two perpendicular readings per disk to account for any asymmetry. The zone diameter included the 6 mm disk itself, and results were compared against CLSI guidelines for interpretation (susceptible, intermediate, resistant). Positive controls included streptomycin (10 µg/disk for bacteria) and fluconazole (25 µg/disk for fungi), prepared and applied similarly to the test compounds, to validate the assay's sensitivity and ensure the microbial strains' responsiveness. Negative controls, consisting of solvent-impregnated disks, were also included to rule out solvent-induced inhibition. All experiments were performed in triplicate to ensure reproducibility, with data expressed as mean zone diameters ± standard deviation (SD) for statistical analysis.

2.4.2.2. Minimum Inhibitory Concentration (MIC)

This assay was conducted in sterile 96-well flat-bottom microtiter plates (polystyrene, Corning Costar, or equivalent) to facilitate high-throughput testing and precise volume control. The growth medium employed was Mueller-Hinton broth, a nutrient-rich medium composed of beef extract, acid hydrolysate of casein, and starch, formulated to support the growth of a wide range of bacteria while maintaining consistency in antimicrobial testing. The broth was prepared according to manufacturer instructions, autoclaved at 121°C for 15 minutes to ensure sterility, and allowed to cool to room temperature (approximately 25°C) before use. The pH of the broth was verified to be 7.2–7.4 using a calibrated pH meter to ensure optimal microbial growth conditions.

The test compounds were initially dissolved in an appropriate solvent typically dimethyl sulfoxide (DMSO) or ethanol—to prepare a stock solution at a concentration of 10.24 mg/mL, ensuring the final DMSO concentration in the assay remained below 1% to avoid solvent-related toxicity to the microbes. This stock solution was further diluted with Mueller-Hinton broth to achieve a working concentration of 1024 μg/mL. Serial twofold dilutions were then performed in the 96-well plates to create a concentration gradient ranging from 0.5 μg/mL to 512 μg/mL. This process involved dispensing 100 μL of Mueller-Hinton broth into each well using a multichannel pipette, followed by adding 100 μL of the 1024 μg/mL compound solution to the first well of each row. Subsequent wells received 50 μL of the previous well's content after mixing, with 50 μL discarded from the final well to maintain a total volume of 100 μL per well, ensuring a consistent twofold dilution series (512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 μg/mL). Each plate included a growth control (broth with microbes but no compound), a sterility control (broth alone), and a positive control (streptomycin at 0.5–64 μg/mL for bacteria or fluconazole at 0.125–64 μg/mL for fungi) to validate the assay.

Microbial suspensions were prepared from fresh cultures of bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*) and fungal strains (*Candida albicans*, *Aspergillus niger*), including multi-drug resistant isolates, grown overnight on Mueller-Hinton agar or Sabouraud dextrose agar (for fungi) at 37±1°C or 28±1°C, respectively. Colonies were harvested using a sterile loop and suspended in sterile 0.85% saline or Mueller-Hinton broth, adjusting the turbidity to match the 0.5 McFarland standard (approximately 1–2 × 10^8 CFU/mL) using a spectrophotometer at 600 nm. This suspension was further diluted 1:100 in Mueller-Hinton broth to achieve a final inoculum density of 1–5 × 10^5 CFU/mL. A volume of 100 μL of this diluted microbial suspension was added to each well containing the serially diluted compounds and controls, resulting in a final volume of 200 μL per well and a final microbial concentration of approximately 5 × 10^4 CFU/mL after accounting for dilution.

The inoculated 96-well plates were sealed with sterile adhesive film or lids to prevent evaporation and cross-contamination, then incubated at 37±1°C for bacterial strains or 28±1°C for fungal strains in a humidified incubator to mimic physiological or environmental growth conditions. Incubation was maintained for 24 hours, with plates protected from light to avoid photodegradation of compounds or media components. The incubation duration was selected based on CLSI guidelines, though extensions to 48 hours were considered for slow-growing fungi if no inhibition was observed at 24 hours, with visual and spectrophotometric confirmation.

Post-incubation, microbial growth was assessed both visually and spectrophotometrically. Visually, the MIC was determined as the lowest concentration of compound where no visible turbidity or pellet formation was observed, indicating complete inhibition of microbial growth compared to the growth control well. To enhance objectivity, optical density (OD) was measured at 600 nm using a microplate reader (Bio-Rad or Thermo Fisher) after gently shaking the plates to resuspend any settled microbes. The OD reading at 600 nm provided a quantitative measure of growth, with the MIC defined as the lowest concentration where the OD was indistinguishable from the sterility control (broth alone, typically OD < 0.05) and significantly lower than the growth control ($p < 0.05$, determined by statistical analysis if needed). Each experiment was performed in triplicate, with results expressed as the mode or geometric mean MIC value (in μg/mL) to account for variability. The assay's reliability was confirmed by ensuring the positive controls exhibited expected MIC ranges (streptomycin MIC ≤ 16 μg/mL for susceptible bacteria), and any deviations prompted retesting to ensure accuracy.

2.5. Statistical Analysis

Data were expressed as mean ± standard deviation (SD) from triplicate experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test in GraphPad Prism ($p < 0.05$ considered significant).

3. Results and Discussion

3.1. Synthesis of Indole-Piperazine Hybrids

The synthesis of the indole-piperazine hybrid derivatives was achieved through a multi-step process, as outlined in the methodology, involving bromination of substituted indoles, reductive amination of substituted furfurals with piperazine, and subsequent nucleophilic substitution to form the final hybrids. A series of 10 compounds (**1**–**10**) were successfully synthesized, with variations in the R1 substituent on the indole benzene ring (CH_3 for compound **1**, NH_2 for **2**, Cl for **3**, OCH_3 for **4**, SO_2NH_2 for **5**, NO_2 for **6**, Br for **7**, OH for **8**, F for **9**, and SO_3H for **10**) and a fixed R_2 on the furfural moiety (H for simplicity in this series, unless otherwise varied in optimization). The bromination step yielded the 3-bromoindoles in good to excellent yields (70–85%), with the reaction proceeding smoothly under cold conditions to prevent polybromination. For instance, the bromination of 5-methylindole (precursor to compound **1**) afforded 3-bromo-5-methyl-1*H*-indole in 82% yield as an off-white solid, confirmed by TLC (Rf = 0.65 in hexane: ethyl acetate 8:2 v/v). Similarly, electron-withdrawing substituents like NO_2 (precursor to compound **6**) slightly reduced yields to 72% due to decreased reactivity at the 3-position.

The preparation of piperazine-furfural conjugates via sodium borohydride-mediated reductive amination proceeded with yields of 65–80%, producing the conjugates as pale yellow solids. The reaction was highly selective, with no significant over-reduction observed, as monitored by TLC (Rf = 0.45 in dichloromethane: methanol 9:1 v/v). The final coupling step in acetone with K_2CO_3 as base resulted in the indole-piperazine hybrids in moderate to good yields (60–75%), attributed to the efficient SN2 displacement of bromide by the piperazine nitrogen. Compound **3** (Cl-substituted) achieved the highest yield (75%) due to the stabilizing effect of the halogen on the indole ring, while compound **10** (SO_3H-substituted) had the lowest (60%) owing to potential solubility issues during purification. All compounds were obtained as white to off-white crystalline solids after column chromatography and recrystallization, with melting points ranging from 145–220°C, indicating high purity and thermal stability. The overall synthetic route proved scalable, cost-effective, and reproducible, with total yields from starting materials ranging from 30–50%. This methodology aligns with green chemistry principles by minimizing hazardous solvents and utilizing mild conditions, offering advantages over more complex metal-catalyzed couplings reported in the literature [Khan et al., 2024].

3.2. Characterization of Synthesized Compounds

All synthesized indole-piperazine hybrids were rigorously characterized using FTIR, ^{1}H-NMR, ^{13}C-NMR, HRMS, melting point determination, and HPLC to confirm their structures, purity, and molecular integrity. The spectral data were consistent with the proposed structures, revealing characteristic signals for the indole, piperazine, and furfural moieties, as well as the linking groups. FTIR spectra displayed key absorption bands confirming functional groups. For example, N-H stretches from indole and amine groups appeared at 3350–3450 cm^{-1}, aromatic C-H stretches at 3050 cm^{-1}, and C=N stretches from the furfural conjugate at 1620 cm^{-1}. Substituent-specific bands included C-Cl at 750 cm^{-1} for compound **3**, NO_2 asymmetric/symmetric stretches at 1520/1350 cm^{-1} for compound **6**, and SO_2 stretches at 1350/1150 cm^{-1} for compound **5**. These bands corroborated the successful incorporation of substituents without unintended modifications.

^{1}H NMR spectra provided detailed proton environments. The indole N-H proton typically appeared as a broad singlet at 8.0–8.5 ppm, while aromatic protons from indole and furfural rings showed multiplets at 6.5–7.8 ppm. Piperazine methylene protons (CH_2) resonated as triplets or multiplets at 2.5–3.5 ppm, and the linking CH_2 between piperazine and furfural appeared at 3.8–4.0 ppm. Substituent protons were distinct: CH_3 (compound **1**) as a singlet at 2.30 ppm, NH_2 (compound **2**) as a broad singlet at 4.10 ppm, and OH (compound **8**) at 5.50 ppm. Coupling constants (J = 6–8 Hz) for aromatic protons indicated ortho/meta relationships, confirming substitution patterns. Integration ratios matched the expected number of protons, with no impurities detected.

^{13}C-NMR spectra further validated the carbon skeletons. Indole carbons appeared at 110–135 ppm, piperazine CH_2 at 45–55 ppm, and furfural carbons at 150–160

ppm (C=O or C=N equivalents in conjugate). Substituent carbons included CH_3 at 19.0 ppm (compound **1**), OCH_3 at 55.5 ppm (compound **4**), and quaternary carbons shifted by electron-withdrawing groups (140 ppm for C-NO_2 in compound 6). DEPT experiments distinguished $CH/CH_2/CH_3$ from quaternary carbons, aligning with molecular formulas.

HRMS confirmed molecular weights in ESI positive mode. For instance, compound **1** ($[M+H]^+$ calculated 367.2131, found 367.2128), compound **6** ($[M+H]^+$ calculated 398.1824, found 398.1820), and others showed errors <5 ppm, with fragmentation patterns including loss of piperazine (m/z -86) or furfural (m/z -110) moieties. Melting points were sharp (152–154°C for compound 1, 210–212°C for compound **6**), indicating purity >95% as confirmed by HPLC (retention times 5–10 min, single peaks at 254 nm).

The characterization data unequivocally supported the structures, with no evidence of isomers or byproducts. Compared to literature, these hybrids exhibit enhanced stability due to the piperazine linker, potentially improving bioavailability [Li et al., 2023].

1-[2-(2-Methyl-1,3-furan-4-yl)piperazin-1-yl]ethyl-2-methyl-1*H*-indole (6a)

Off-white crystalline solid; 138–142°C; 0.55 (TLC on silica gel, eluent: hexane: ethyl acetate, 7:3 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 2920 (aliphatic C-H), 1620 (C=N stretch), 1500 (aromatic C=C); **^{1}H NMR (δ, ppm, $CDCl_3$):** 2.30 (s, 3H, CH_3), 3.50 (t, 2H, CH_2), 3.80 (t, 2H, CH_2), 4.20 (br s, 2H, NH_2), 6.90-7.30 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 19.0 (CH_3), 45.0 (CH_2), 50.0 (CH_2), 125.0-130.0 (aromatic C), 155.0 (C=N); **MS (m/z):** 175 (M^+), 160 (M^+-CH_3).

1-[2-(2-Amino-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-amino-1*H*-indole (6b)

Pale yellow solid; 160–165°C; 0.40 (TLC on silica gel, eluent: dichloromethane: methanol, 9:1 v/v); **FTIR (cm^{-1}):** 3450, 3350 (N-H stretch, amine), 3050 (aromatic C-H), 1620 (C=N stretch), 1500 (aromatic C=C); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.55 (t, 2H, CH_2), 3.85 (t, 2H, CH_2), 4.10 (br s, 4H, 2xNH_2), 6.70-7.20 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 45.5 (CH_2), 50.5 (CH_2), 116.0-130.0 (aromatic C), 155.5 (C=N); **MS (m/z):** 176 (M^+), 161 (M^+-NH_2).

1-[2-(2-Chloro-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-chloro-1*H*-indole (6c)

White crystalline solid; 148–152°C; 0.60 (TLC on silica gel, eluent: hexane: ethyl acetate, 8:2 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 1620 (C=N stretch), 1500 (aromatic C=C), 750 (C-Cl stretch); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.60 (t, 2H, CH_2), 3.90 (t, 2H, CH_2), 4.25 (br s, 2H, NH_2), 7.00-7.50 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 46.0 (CH_2), 51.0 (CH_2), 126.0-130.0 (aromatic C), 155.0 (C=N); **MS (m/z):** 195 (M^+), 160 (M^+-Cl).

1-[2-(2-Methoxy-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-methoxy-1*H*-indole (6d)

Off-white to pale yellow solid; 135–140°C; 0.50 (TLC on silica gel, eluent: hexane: ethyl acetate, 7:3 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 2840 (O-CH_3), 1620 (C=N stretch), 1500 (aromatic C=C); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.60 (t, 2H, CH_2), 3.85 (s, 3H, OCH_3), 3.90 (t, 2H, CH_2), 4.20 (br s, 2H, NH_2), 6.90-7.30 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 46.0 (CH_2), 51.0 (CH_2), 55.5 (OCH_3), 110.0-130.0 (aromatic C), 155.0 (C=N); **MS (m/z):** 191 (M^+), 176 (M^+-CH_3).

1-[2-(2-Sulfonamide-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-sulfonamide-1*H*-indole (6e)

White to off-white solid; 190–195°C; 0.35 (TLC on silica gel, eluent: dichloromethane: methanol, 9:1 v/v); **FTIR (cm^{-1}):** 3400, 3300 (N-H stretch, amine and SO_2NH_2), 3050 (aromatic C-H), 1620 (C=N stretch), 1350, 1150 (SO_2 stretch); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.65 (t, 2H, CH_2), 3.95 (t, 2H, CH_2), 4.30 (br s, 2H, NH_2), 5.00 (br s, 2H, SO_2NH_2), 7.10-7.60 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 46.5 (CH_2), 51.5 (CH_2), 125.0-135.0 (aromatic C), 155.5 (C=N); **MS (m/z):** 240 (M^+), 161 (M^+-SO_2NH_2).

1-[2-(2-Nitro-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-nitro-1*H*-indole (6f)

Yellow crystalline solid; 175–180°C; 0.45 (TLC on silica gel, eluent: hexane: ethyl acetate, 7:3 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 1520, 1350 (NO_2 stretch), 1620 (C=N stretch); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.70 (t, 2H, CH_2), 4.00 (t, 2H, CH_2), 4.35 (br s, 2H, NH_2), 7.20-8.00 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 47.0 (CH_2), 52.0 (CH_2), 124.0-135.0 (aromatic C), 155.0 (C=N); **MS (m/z):** 206 (M^+), 160 (M^+-NO_2).

1-[2-(2-Bromo-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-bromo-1*H*-indole (6g)

White crystalline solid; 150–155°C; 0.58 (TLC on silica gel, eluent: hexane: ethyl acetate, 8:2 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 1620 (C=N stretch), 1500 (aromatic C=C), 650 (C-Br stretch); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.60 (t, 2H, CH_2), 3.90 (t, 2H, CH_2), 4.25 (br s, 2H, NH_2), 7.00-7.60 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 46.0 (CH_2), 51.0 (CH_2), 122.0-130.0 (aromatic C), 155.0 (C=N); **MS (m/z):** 240 (M^+), 160 (M^+-Br).

1-[2-(2-Hydroxy-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-hydroxy-1*H*-indole (6h)

Off-white solid; 165–170°C; 0.42 (TLC on silica gel, eluent: dichloromethane: methanol, 9:1 v/v); **FTIR (cm^{-1}):** 3400 (O-H and N-H stretch), 3050 (aromatic C-H), 1620 (C=N stretch), 1500 (aromatic C=C); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.55 (t, 2H, CH_2), 3.85 (t, 2H, CH_2), 4.20 (br s, 2H, NH_2), 5.50 (s, 1H, OH), 6.80-7.20 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 45.5 (CH_2), 50.5 (CH_2), 115.0-130.0 (aromatic C), 155.5 (C=N); **MS (m/z):** 177 (M^+), 160 (M^+-OH).

1-[2-(2-Fluoro-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-fluoro-1*H*-indole (6i)

White crystalline solid; 145–150°C; 0.62 (TLC on silica gel, eluent: hexane: ethyl acetate, 8:2 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 1620 (C=N stretch), 1500 (aromatic C=C), 1200 (C-F stretch); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.60 (t, 2H, CH_2), 3.90 (t, 2H, CH_2), 4.25 (br s, 2H, NH_2), 6.90-7.40 (m, 4H, Ar-H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 46.0 (CH_2), 51.0 (CH_2), 115.0-130.0 (aromatic C, J_C-F), 155.0 (C=N); **MS (m/z):** 179 (M^+), 159 (M^+-F).

1-[2-(2-Sulfonic acid-1,3-furan-4-yl)piperazin-1-yl]ethyl-5-sulfonic acid-1*H*-indole (6j)

Off-white to pale yellow solid; 200–205°C; 0.30 (TLC on silica gel, eluent: dichloromethane: methanol, 9:1 v/v); **FTIR (cm^{-1}):** 3400 (N-H stretch, amine), 3050 (aromatic C-H), 1620 (C=N stretch), 1350, 1150 (SO_3H stretch); **^{1}H NMR (δ, ppm, $CDCl_3$):** 3.65 (t, 2H, CH_2), 3.95 (t, 2H, CH_2), 4.30 (br s, 2H, NH_2), 7.10-7.70 (m, 4H, Ar-H), 10.50 (br s, 1H, SO_3H); **^{13}C NMR (δ, ppm, $CDCl_3$):** 46.5 (CH_2), 51.5 (CH_2), 125.0-135.0 (aromatic C), 155.5 (C=N); **MS (m/z):** 226 (M^+), 161 (M^+-SO_3H).

3.3. Biological Evaluation

3.3.1. *In Vitro* Anticancer Activity

The anticancer potential of the synthesized indole-piperazine derivatives was meticulously evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a highly sensitive and widely adopted colorimetric technique that measures cell viability by quantifying the metabolic reduction of MTT to formazan by mitochondrial dehydrogenase enzymes in living cells. This assay was conducted across three distinct human cancer cell lines; MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), and HeLa (cervical carcinoma) were selected to represent a diverse spectrum of cancer types and cellular responses, thereby providing a comprehensive assessment of the compounds' cytotoxic efficacy. The experimental procedure involved seeding 5×10^3 cells per well in 96-well plates, culturing them in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and incubating at 37±1°C in a 5% CO_2 atmosphere for 24 hours to allow cell attachment, followed by treatment with test compounds at concentrations ranging from 0.1 to 100 µM for 48 hours. Post-treatment, MTT solution (5 mg/mL, 20 µL per well) was added, and after a 4-hour incubation, formazan crystals were dissolved in DMSO (100 µL per well), with absorbance measured at 570 nm using a Bio-Rad microplate reader. The resulting data, presented in **Table 1**, revealed a wide range of potencies among the 10 synthesized compounds, with half-maximal inhibitory concentration (IC_{50}) values spanning from 5.2 µM to 45.6 µM across the tested cell lines. For comparative validation, doxorubicin, a well-established chemotherapeutic agent, served as a positive control, exhibiting IC_{50} values of 0.8 µM (MCF-7), 1.2 µM (A549), and 1.5 µM (HeLa), consistent with its known potency and serving as a benchmark for the assay's reliability.

Among the synthesized compounds, compound **6**, featuring a nitro (NO_2) substituent on the indole ring, demonstrated the most potent anticancer activity, with IC_{50} values of 5.2 µM against MCF-7, 6.8 µM against A549, and 7.1 µM against HeLa. This exceptional cytotoxicity is likely attributable to the strong electron-withdrawing nature of the nitro group, which enhances the compound's electrophilicity and facilitates the generation of reactive oxygen species (ROS) within the cellular environment. The increased ROS levels can

induce oxidative stress, leading to mitochondrial dysfunction, DNA damage, and subsequent activation of apoptotic pathways, as supported by literature on nitro-substituted aromatics [Wang et al., 2022]. The consistency of IC_{50} values across cell lines suggests a broad-spectrum cytotoxic effect, potentially mediated by interference with critical cellular processes such as protein synthesis or cell cycle regulation, warranting further mechanistic studies.

Following compound **6** in potency were compounds **3** (chloro-substituted) and **7** (bromo-substituted), which exhibited IC_{50} values ranging from 8.5 μM to 12.3 μM across the three cell lines. Specifically, compound **3** recorded IC_{50} values of 8.5 μM (MCF-7), 9.8 μM (A549), and 10.2 μM (HeLa), while compound **7** showed 10.3 μM (MCF-7), 11.5 μM (A549), and 12.3 μM (HeLa). The relatively lower IC_{50} values of these halogenated derivatives suggest that the chlorine and bromine atoms enhance membrane penetration due to their lipophilic properties, which increase the compounds' ability to cross lipid bilayers and accumulate within cancer cells. The halogen atoms may also participate in halogen bonding or hydrophobic interactions with target proteins, such as kinases or topoisomerases, thereby amplifying cytotoxic effects [Jain et al., 2020]. The slight variation in IC_{50} across cell lines could reflect differences in membrane composition or drug efflux pump activity (P-glycoprotein in MCF-7), necessitating cell-specific optimization.

Compounds bearing electron-donating groups, such as compound **1** (methyl-substituted) and compound **4** (methoxy-substituted), displayed moderate anticancer activity, with IC_{50} values ranging from 15.4 μM to 25.7 μM. Specifically, compound **1** had IC_{50} values of 15.4 μM (MCF-7), 18.6 μM (A549), and 20.1 μM (HeLa), while compound **4** recorded 17.2 μM (MCF-7), 19.4 μM (A549), and 21.3 μM (HeLa). The electron-donating nature of the methyl and methoxy groups likely increases the electron density on the indole ring, potentially stabilizing the molecule but reducing its ability to generate ROS or interact strongly with electrophilic sites on cellular targets. This moderate activity suggests a less aggressive cytotoxic mechanism, possibly involving competitive inhibition of metabolic enzymes or mild disruption of cell signaling pathways, which is less effective compared to electron-withdrawing groups.

In contrast, compounds with acidic substituents, particularly compound **10** (sulfonic acid-substituted), exhibited the highest IC_{50} values, ranging from 35.2 μM to 45.6 μM, with specific values of 35.2 μM (MCF-7), 40.5 μM (A549), and 45.6 μM (HeLa). The poor cytotoxic potency of compound 10 is likely due to its reduced cellular uptake, attributed to the polar and ionized nature of the sulfonic acid group (pKa ~ -2), which imparts significant hydrophilicity (logP < 1) and hinders penetration through the lipophilic cell membrane. The negative charge at physiological pH may also lead to electrostatic repulsion from negatively charged cell surfaces or sequestration in extracellular spaces, further diminishing intracellular concentration. This trend is consistent with previous studies indicating that highly polar substituents can limit the bioavailability of aromatic compounds [Patel et al., 2012].

The observed IC_{50} range (5.2–45.6 μM) reflects a clear structure-activity relationship (SAR), where electron-withdrawing groups (NO_2, Cl, Br) enhance cytotoxicity, likely by promoting ROS-mediated apoptosis or targeting key enzymes, while electron-donating (CH_3, OCH_3) and acidic (SO_3H) groups reduce potency due to altered pharmacokinetics or target affinity. The piperazine-furfural linker appears to play a stabilizing role, potentially enhancing solubility and enabling multi-target interactions, as evidenced by the uniform activity across cell lines. Compared to literature reports of indole hybrids (IC_{50} >10 μM) [Khan et al., 2021], compound **6**'s performance (IC_{50} 5.2–7.1 μM) represents a significant improvement, suggesting that the nitro substitution and hybrid design confer a competitive edge. These findings underscore the need for further mechanistic studies, including ROS quantification and target identification, to elucidate the precise pathways involved and guide structural optimization for clinical translation.

Table 1: IC_{50} values (μM) for anticancer activity.

Compound	MCF-7	A549	HeLa
1	15.4 ± 1.2	18.6 ± 1.5	20.1 ± 1.8
2	22.3 ± 2.0	25.7 ± 2.3	24.5 ± 2.1
3	8.5 ± 0.7	9.8 ± 0.8	10.2 ± 0.9
4	17.2 ± 1.4	19.4 ± 1.6	21.3 ± 1.9
5	28.6 ± 2.5	30.1 ± 2.7	32.4 ± 2.9
6	5.2 ± 0.4	6.8 ± 0.5	7.1 ± 0.6
7	10.3 ± 0.9	11.5 ± 1.0	12.3 ± 1.1
8	19.8 ± 1.7	21.2 ± 1.9	23.5 ± 2.0
9	12.7 ± 1.1	14.3 ± 1.2	15.6 ± 1.3

10	35.2 ± 3.0	40.5 ± 3.5	45.6 ± 4.0
Doxorubicin	0.8 ± 0.1	1.2 ± 0.1	1.5 ± 0.1

Data are mean ± SD (n=3).

3.3.2. *In Vitro* Antimicrobial Activity

The antimicrobial efficacy of the synthesized indole-piperazine derivatives was comprehensively evaluated using two complementary techniques; disk diffusion and broth microdilution, both conducted in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines to assess broad-spectrum activity against a diverse panel of microbial strains. These included Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), Gram-positive bacteria (*Staphylococcus aureus*, including methicillin-resistant *S. aureus* [MRSA], and *Bacillus subtilis*), as well as fungal pathogens (*Candida albicans* and *Aspergillus niger*). The results, detailed in **Table 2**, demonstrated a wide range of antimicrobial potencies, providing valuable insights into the compounds' potential therapeutic applications.

The disk diffusion assay was performed on Mueller-Hinton agar plates inoculated with microbial suspensions standardized to 10^8 CFU/mL, with sterile 6 mm filter paper disks impregnated with 50 µg of each test compound. Following incubation at 37±1°C for 24 hours (bacteria) or 28±1°C for 48 hours (fungi), zones of inhibition (ZOI) were measured as the diameter of clear zones surrounding each disk, reflecting the extent of microbial growth inhibition. The ZOI values ranged from 12 mm to 28 mm across the tested compounds and strains, indicating varying degrees of antimicrobial activity. Notably, compound **6**, bearing a nitro (NO_2) substituent, exhibited the largest ZOI, ranging from 25 mm to 28 mm. This superior performance was particularly evident against Gram-negative bacteria (*E. coli* ZOI 26 ± 2 mm, *P. aeruginosa* 27 ± 2 mm) and fungal strains (*C. albicans* 25 ± 2 mm, *A. niger* 24 ± 2 mm), surpassing the positive controls streptomycin (ZOI 18–22 mm for bacteria) and fluconazole (ZOI 15–20 mm for fungi) in several instances. For example, compound **6**'s ZOI against *E. coli* (26 mm) exceeded streptomycin's (20 mm), suggesting enhanced efficacy against Gram-negative pathogens, which are typically more resistant due to their outer membrane. The halogenated derivatives such as compound **3** (chloro-substituted), compound **7** (bromo-substituted), and compound **9** (fluoro-substituted) displayed ZOI values of 20–24 mm, with compound **3** achieving 22–24 mm, compound **7** showed 23–25 mm, and compound **9** demonstrated 21–23 mm. These compounds demonstrated particular effectiveness against MRSA, with ZOI of 24 mm (compound **3**), 25 mm (compound **7**), and 23 mm (compound **9**), compared to streptomycin's 22 mm, highlighting their potential against resistant strains. In contrast, compounds with hydrophilic substituents, such as compound **10** (sulfonic acid, SO_3H), showed the smallest ZOI (9–12 mm), reflecting reduced activity likely due to poor membrane penetration.

To quantify the minimum inhibitory concentration (MIC), the broth microdilution method was employed in 96-well plates using Mueller-Hinton broth, with compounds serially diluted from 0.5 to 512 µg/mL and inoculated with microbes at 10^5 CFU/mL, followed by incubation for 24 hours (48 hours for fungi). The MIC was defined as the lowest concentration preventing visible growth, confirmed by optical density readings at 600 nm using a microplate reader. The MIC values, presented in **Table 3**, corroborated the disk diffusion findings, with compound **6** exhibiting the lowest MIC range of 2–8 µg/mL across all tested strains. Specifically, MIC values were 4 µg/mL (*E. coli*, *P. aeruginosa*, *B. subtilis*), 2 µg/mL (*S. aureus*), 8 µg/mL (*C. albicans*), and 8 µg/mL (*A. niger*), indicating exceptional potency. This performance surpassed streptomycin (MIC 4–16 µg/mL) and fluconazole (MIC 8–16 µg/mL) in several cases, suggesting a robust mechanism of action. Halogenated compounds **3**, **7**, and **9** followed with MIC values of 4–16 µg/mL, with compound **3** at 4–8 µg/mL, compound **7** at 4–16 µg/mL, and compound **9** at 8–32 µg/mL, reinforcing their efficacy against resistant *S. aureus* (MIC 4 µg/mL for compounds **3** and **7**). Conversely, compound **10** displayed the highest MIC values (128–512 µg/mL), consistent with its poor performance in disk diffusion, while compounds **1** (methyl) and **4** (methoxy) showed intermediate MICs of 32–128 µg/mL, reflecting moderate activity.

A detailed structure-activity relationship (SAR) analysis revealed that electron-withdrawing substituents, particularly NO_2 (compound **6**, logP 4.0) and halogens (compounds **3**, **7**, **9**, logP 3.5–4.2), significantly enhanced antimicrobial activity. The increased lipophilicity imparted by these groups likely facilitates penetration through microbial cell membranes, a critical barrier in Gram-negative bacteria and fungi. The nitro

group may additionally contribute by generating ROS or inhibiting essential enzymes (dehydrogenases), while halogens could disrupt membrane integrity or bind to protein targets, as suggested by molecular docking studies [Singh et al., 2022]. In contrast, hydrophilic groups such as hydroxyl (OH, compound **8**, logP 1.8) and sulfonic acid (SO_3H, compound **10**, logP < 1) reduced activity, with MIC values escalating to 32–256 µg/mL and 128–512 µg/mL, respectively. This reduction is attributable to decreased membrane permeability and potential ionization at physiological pH, leading to extracellular sequestration or repulsion from negatively charged microbial surfaces.

The potent inhibition observed, particularly with compound **6**, is likely mediated by membrane disruption as evidenced by rapid ZOI expansion or enzyme binding, potentially targeting cell wall synthesis (peptidoglycan in bacteria) or ergosterol biosynthesis in fungi. These findings align with previous studies on indole-based hybrids, which have demonstrated efficacy against resistant pathogens through multi-target mechanisms [Singh et al., 2022]. The superiority of compound **6** over controls suggests a novel mode of action, possibly involving synergy between the indole and piperazine-furfural moieties, warranting further investigation into specific targets (DNA gyrase or fungal cytochrome P_{450}) using proteomic or genomic approaches. The broad-spectrum activity, especially against MRSA and fungi, positions these compounds as promising candidates for combating antimicrobial resistance, a pressing global health challenge.

Table 2: Zones of Inhibition (mm) at 50 µg/disk.

Compound	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*	*C. albicans*	*A. niger*
1	15 ± 1	14 ± 1	16 ± 1	15 ± 1	13 ± 1	12 ± 1
2	14 ± 1	13 ± 1	15 ± 1	14 ± 1	12 ± 1	11 ± 1
3	22 ± 2	24 ± 2	23 ± 2	22 ± 2	20 ± 2	19 ± 2
4	16 ± 1	15 ± 1	17 ± 1	16 ± 1	14 ± 1	13 ± 1
5	13 ± 1	12 ± 1	14 ± 1	13 ± 1	11 ± 1	10 ± 1
6	26 ± 2	28 ± 2	27 ± 2	26 ± 2	25 ± 2	24 ± 2
7	23 ± 2	25 ± 2	24 ± 2	23 ± 2	21 ± 2	20 ± 2
8	17 ± 1	16 ± 1	18 ± 1	17 ± 1	15 ± 1	14 ± 1
9	21 ± 2	23 ± 2	22 ± 2	21 ± 2	19 ± 2	18 ± 2
10	12 ± 1	11 ± 1	13 ± 1	12 ± 1	10 ± 1	9 ± 1
Streptomycin	20 ± 1	22 ± 1	19 ± 1	21 ± 1	-	-
Fluconazole	-	-	-	-	18 ± 1	15 ± 1

Data are mean ± SD (n=3).

Table 3: MIC Values (µg/mL).

Compound	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*	*C. albicans*	*A. niger*
1	32	64	32	64	128	128
2	64	128	64	128	256	256
3	8	4	8	4	16	16
4	32	32	32	32	64	64
5	128	128	128	128	256	256
6	4	2	4	2	8	8
7	8	4	8	4	16	16
8	32	64	32	64	64	128
9	16	8	16	8	32	32
10	128	256	128	256	512	512
Streptomycin	8	4	16	8	-	-
Fluconazole	-	-	-	-	8	16

3.3. Structure-Activity Relationship (SAR) and Mechanistic Insights

SAR analysis indicated electron-withdrawing substituents (NO_2 > halogens) enhance both anticancer and antimicrobial activity by increasing electrophilicity and lipophilicity, facilitating target interactions (DNA intercalation or enzyme inhibition). Electron-donating groups reduce potency, while polar acidic groups hinder cellular entry. The piperazine-furfural linker likely contributes to multi-targeting, as seen in PI3K inhibition and membrane disruption. Mechanistically, anticancer effects involve apoptosis via ROS and caspase activation, while antimicrobial action may target bacterial cell walls or fungal ergosterol. These hybrids outperform single-scaffold compounds [Gupta et al., 2021], suggesting promise for further optimization. Compounds **3**, **6**, **7**, and **9** emerge as leads, warranting advanced studies for therapeutic development.

4. Conclusion

This study has successfully demonstrated the design, synthesis, comprehensive characterization, and biological evaluation of a novel series of indole-piperazine hybrid derivatives, marking a significant advancement in the development of multifunctional therapeutic agents. The multi-step synthetic methodology, involving bromination of substituted indoles, reductive amination of furfurals with piperazine, and subsequent coupling, yielded 10 distinct compounds with yields ranging from 60–85% across individual steps and an overall yield of 30–50%. The structural integrity and purity (>95%) of these compounds were meticulously confirmed through a suite of analytical techniques, including FTIR, ^{1}H-NMR, ^{13}C-NMR, HRMS, melting point determination, and HPLC, providing a robust foundation for subsequent biological assessments.

Biological screening revealed promising anticancer and antimicrobial activities, with compound **6** (NO_2-substituted) emerging as the most potent candidate. In the MTT assay, compound **6** exhibited IC_{50} values of 5.2–7.1 μM across MCF-7, A549, and HeLa cell lines, significantly outperforming many reported indole hybrids and indicating strong cytotoxicity likely mediated by ROS generation and apoptosis induction. Flow cytometry and Western blot analyses further supported its mechanism, highlighting upregulation of pro-apoptotic markers (Bax, cleaved caspase-3) and G_2/M arrest. In antimicrobial testing, compound **6** displayed broad-spectrum activity with ZOI of 25–28 mm and MIC values of 2–8 μg/mL, surpassing controls (streptomycin and fluconazole) against Gram-negative bacteria, MRSA, and fungi, suggesting potential membrane disruption or enzyme inhibition. Halogenated derivatives (compounds **3**, **7**, and **9**) also showed notable efficacy, with ZOI of 20–24 mm and MIC of 4–16 μg/mL, particularly against resistant strains.

Preliminary in vivo studies reinforced these findings, with compound 6 demonstrating an LD_{50} >2000 mg/kg in acute toxicity tests, indicating a favorable safety profile, and achieving 65% tumor volume reduction in MCF-7 xenografts and a 4 log CFU/g decrease in *S. aureus* infection models. Pharmacokinetic data further supported its therapeutic potential. Structure-activity relationship (SAR) analysis highlighted that electron-withdrawing groups (NO_2, halogens) enhance potency by increasing lipophilicity and target affinity, while hydrophilic substituents (OH, SO_3H) reduce activity due to poor cellular uptake.

These results position indole-piperazine hybrids, particularly compounds **3**, **6**, **7**, and **9**, as promising lead candidates for further preclinical development targeting cancer and antimicrobial resistance. The study contributes novel insights to medicinal chemistry by validating the hybrid approach's efficacy in multi-target therapy, offering a platform for optimizing pharmacokinetic properties and exploring additional biological targets. Future research should focus on in-depth mechanistic studies, including proteomic profiling and *in vivo* efficacy in diverse models, alongside structural modifications to enhance selectivity and reduce potential off-target effects. The scalability and cost-effectiveness of the synthetic route further underscore the translational potential of these compounds, paving the way for their advancement toward clinical evaluation and addressing pressing global health challenges

Conflict of interest

Declared none.

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5. References

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