

# Design, Biological Evaluation, and Zebrafish Embryo Toxicity of a Novel Pyrazoline Derivative

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## ABSTRACT

**Background:** Pyrazoline derivatives have significant medicinal use for their diverse pharmacological activities. Zebrafish embryos are mostly used in toxicity studies due to its transparency, rapid development and genetic similarity to humans. Fish Embryo Toxicity Test provides rapid, ethical and resource efficient screening. This study aimed to synthesize and evaluate a novel pyrazoline derivative for antibacterial, cytotoxic, and toxicity properties. **Materials and Methods:** The compound 3-(1,3-diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol, was synthesized through a multi-step Mannich reaction and characterized using FT-IR, NMR and Mass spectrometry. Drug likeness and pharmacokinetic parameters were predicted with Molinspiration, OSIRIS, and SwissADME software's. Molecular docking targeted Topoisomerase II and DNA gyrase. Antibacterial activity was tested against *E. coli*, *P. aeruginosa* and *S. aureus*. Cytotoxicity effect against Dalton's Lymphoma Ascites cells and Zebrafish Embryo Toxicity (OECD 236) was evaluated for its toxicity. **Results:** Computational prediction shows favourable drug-likeness and adherence to Lipinski's five rules. Docking studies demonstrated substantial binding affinities: -8.14 kcal/mol and -7.28 kcal/mol for Topoisomerase II and DNA gyrase respectively. The compound exhibited potent anti-bacterial activity with inhibition zone ranging from 12 to 17 mm at 100 µg/mL. Cytotoxicity assay revealed dose-dependent increase in cell death, reaching 86.3±2.0% at 200 µg/mL. Zebrafish embryo toxicity assay revealed developmental abnormalities at 50 µg/mL and 25 µg/mL, with an LC<sub>50</sub> of 18.75 µg/mL. **Conclusion:** The compound exhibited favourable pharmacokinetic properties, suggesting its potential as an antibacterial and cytotoxic agent. However, Zebrafish Embryo toxicity highlights the need for further development and in-depth evaluation before it can as a therapeutic candidate.

**Keywords:** Characterization, Docking scores, Inhibition zone, Cytotoxicity, Fish embryos.

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## INTRODUCTION

Pyrazoline derivatives are a class of compound of great interest due to their diverse biological properties and pharmacological activity. Their unique properties make them highly valuable in drug design and development, while their broad synthetic versatility extends their applications across pharmaceuticals, agrochemicals, and materials science (Turkan *et al.*, 2019; Kumar *et al.*, 2009). They exhibit pharmacological properties, including anti-bacterial, anti-microbial, anti-cancer, CNS activity, anti-inflammatory, antioxidant, anti-tubercular, anti-viral, anti-hyperglycaemia, anti-convulsant and anti-depressant activity (Nehra *et al.*, 2020; Ravindar *et al.*, 2023; Song *et al.*, 2020a).

Spectroscopic techniques such as FT-IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, and Mass spectrometry provide rapid and reliable characterization of chemical compounds (Mir *et al.*, 2020). Molecular docking is a well-established *in silico* structure-based approach used in early-stage drug development to identify the new therapeutic compounds, predict ligand-target interaction at molecular level and understand the structure-activity relationship (Pinzi and Rastelli, 2019; Ferreira *et al.*, 2015a). Bactericidal agents eliminate pathogenic bacteria and may help reduce bacterial plaque in the environment. However, not all antibacterial agents possess specific anti-plaque properties. The production and utilization of antibacterial biomaterials in medicine are advancing in various implants, restorative materials, denture-based materials, and tissue conditioners with antibacterial properties were being manufactured and marketed (Kaur and Liu, 2016; Kharaeva *et al.*, 2020). Cytotoxicity refers to the harmful effects a substance can have on cells and is a key measure for evaluating the safety of drugs and therapies. Cytotoxicity assessment plays a critical



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role in cancer treatment, immunology, toxicology, and materials research by ensuring the safety and guiding the risk assessment of novel compounds (Riss *et al.*, 2019).

Zebrafish (*Danio rerio*) is a widely used model for toxicity screening due to their transparency, rapid development and genetic similarity to humans. Fish embryos are valuable in toxicity studies owing to their sensitivity towards environmental changes (Rawlings *et al.*, 2019). Fish Embryo Toxicity (FET) test offers an efficient alternative to traditional animal testing, providing rapid results, reduced resource consumption, and aligning with ethical considerations for early-stage toxicity screening (Braunbeck *et al.*, 2015) (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). The FET test is primarily used to determine the Lethal Concentration (LC<sub>50</sub>) of a compound, and assesses its effects on fish embryos to evaluate potential environmental and health risks. It is increasingly recommended as an alternative to Acute Fish Toxicity tests (AFT), particularly when AFT limit tests indicate toxicity. Comparison studies have demonstrated a strong correlation between FET and AFT outcomes, with a meta-analysis suggesting that narcotic substances exhibit comparable sensitivity in both tests. The main differences are attributed to species variability in AFT (Rawlings *et al.*, 2019; Dang *et al.*, 2017; Birke and Scholz, 2019). The FET test is now a mandatory component of ecotoxicity testing requirements and is widely endorsed for developing toxicological profiles of chemical derivatives (Birke and Scholz, 2019; Fekadu *et al.*, 2022). Importantly, using only embryos, eliminating the need to sacrifice adult fish, which aligning with ethical considerations (Dang *et al.*, 2017). The current study evaluates both the therapeutic efficacy and toxicity profile of the molecule to establish its safety before preclinical studies. The *in silico* and *in vitro* screening, along with the use of zebrafish as an alternative model organism, provides insights into the safety, biological efficacy, and developmental toxicity of the novel synthesized compound (Braunbeck *et al.*, 2015; Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). In this study, a novel pyrazoline derivative, 3-(1,3-diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol, was synthesized and structurally characterized by spectroscopy studies. Its antibacterial and cytotoxic activities were assessed through *in vitro* assays, while its toxicity profile was evaluated through LC<sub>50</sub> determination using the Zebra FET assay as per OECD Guideline 236. Molecular docking and Qualitative Structural Activity Relationship (QSAR) analyses were undertaken to investigate the compound's binding affinity toward key enzymes such as DNA gyrase and Topoisomerase II, providing meaningful insights into its predicted biological activity.

## MATERIALS AND METHODS

### Reagents

Analytical grade chemicals (HPLC grade): Vanillin, Acetophenone, Aniline, Guanidine Hydrochloride, Acetone,

Ethanol, Hydroxylamine Hydrochloride, 40% NaOH Solution from India Mart and Yarrow Chem Products were purchased and used without any further purification.

### Chemistry

A  $\beta$ -amino carbonyl intermediate was synthesized via a Mannich-type condensation of aromatic ketones, vanillin, and aniline using guanidine hydrochloride under solvent-free conditions with stirring at room temperature. The crude product was then purified by recrystallization. Subsequently, this intermediate was reacted with hydroxylamine hydrochloride in ethanol, with 40% NaOH added dropwise, and refluxed for 4 hr. Following workup, the target pyrazoline derivative, 3-(1,3-Diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol, was obtained. Analytical TLC was performed on pre-coated sheets of silica gel G/UV-254 of 0.2 mm thickness using analytical grade solvent and visualizes with UV Light (Song *et al.*, 2020a).

### Materials

Preliminary QSAR study was performed to identify the structural relationships between chemical and biological properties of a compound by using Molinspiration (Khan *et al.*, 2017). Toxicity was assessed for the synthesized compound using OSIRIS software (Mohammed *et al.*, 2022; Rajalakshmi *et al.*, 2021; Kamboj *et al.*, 2023). ADME properties were also determined to explore the pharmacokinetic properties by using Swiss ADME software (Fekadu *et al.*, 2022; Daina *et al.*, 2017). The compound's melting point was assessed using a capillary method with the aid of a melting point determination apparatus. IR spectra were determined by KBr pellet method with FT-IR and <sup>1</sup>HNMR and <sup>13</sup>CNMR spectra were acquired on a Bruker 500 MHz spectrometer, while mass spectra were recorded using mass spectrometry (Morales *et al.*, 2024; Cerecedo-Sáenz *et al.*, 2022; Avram *et al.*, 2021). The synthesized compound was docked on the target protein by using Auto Dock version 4.2 against target protein molecules for toxicity (Araujo-Lima *et al.*, 2023; Cerecedo-Sáenz *et al.*, 2022). The compound was screened for anti-bacterial activity using Agar well diffusion method that measures the Inhibition Zone (IZ) in mm. Further, the compound was assessed by studied for the short-term *In vitro* cytotoxicity test against Dalton's Lymphoma Ascites (DLA) cells to determine the cell viability (Jędrzejczyk *et al.*, 2022; Li *et al.*, 2015). Lethal Dose (LC<sub>50</sub>) for the test compound was determined by Fish Embryo Toxicity (FET) Test performed on Zebrafish as the model organism.

### Synthesis

The novel pyrazoline derivative was synthesized using a multi-step Mannich reaction starting from a  $\beta$ -amino carbonyl compound. A mixture of aromatic ketones (2.2 mmol), vanillin (2.0 mmol), and aniline (2.0 mmol) was treated with guanidine hydrochloride

(10 mol%) under solvent-free conditions at room temperature. The reaction mixture was stirred for 4 hr with the help of magnetic stirrer, and the precipitated solid was collected by filtration. The solid was washed with water to remove any unreacted guanidine hydrochloride, which was soluble in water. The crude product was then purified by recrystallization from a mixture of acetone and ethanol in the ratio (2:3) to obtain the pure intermediate compound which was mentioned in the scheme.

In the next step, the purified intermediate compound was dissolved in (10 mL) ethanol along with 0.05 mol of hydroxylamine hydrochloride. A 40% NaOH solution was added dropwise with constant stirring. The reaction was maintained under reflux conditions on a water bath for 4 hr, and its completion was monitored by TLC using hexane: ethyl acetate (7:3) solvent system. After the reaction was complete, the mixture was allowed to cool to room temperature and then poured into ice-cold water. The resulting precipitate was neutralized with N/10 HCl, filtered, and dried to yield the desired product, 3-(1, 3-Diphenyl-4, 5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol.

### ***In silico* QSAR, Toxicological and ADME Properties**

A preliminary QSAR analysis was conducted using Molinspiration software to explore the structural relationship between the compound's chemical features and its biological activity, as well as to evaluate compliance with Lipinski's rule of five (Araujo-Lima *et al.*, 2023). Toxicity predictions for the synthesized compound were assessed using OSIRIS software (Mohammed *et al.*, 2022; Rajalakshmi *et al.*, 2021). Additionally, the compound's structure was converted into its Simplified Molecular-Input Line-Entry System (SMILES) format and submitted to the Swiss ADME tool (Morales *et al.*, 2024) to estimate *in silico* pharmacokinetic parameters and other molecular properties.

### **Characterization**

The synthesized novel pyrazoline compound, 3-(1,3-Diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol was characterized using a range of analytical techniques. Its melting point was determined using a capillary method with a standard melting point apparatus. Fourier-Transform Infrared (FT-IR) spectroscopy, performed using the KBr pellet method, was employed to identify the functional groups present in the compound (Fekadu *et al.*, 2022; Morales *et al.*, 2024; Cerecedo-Sáenz *et al.*, 2022; Avram *et al.*, 2021). Nuclear magnetic resonance analysis, including both <sup>1</sup>H NMR and <sup>13</sup>C NMR, was performed on a high-resolution Bruker 500 MHz spectrometer to elucidate the structural features of the synthesized compound (Breindel *et al.*, 2019). Furthermore, mass spectrometry was employed to determine the molecular weight and fragmentation pattern of the target molecule (Bouillon, 2016).

### **Docking Study**

The binding interaction of the obtained pyrazoline derivative to DNA Gyrase (PDB Id-5MMO) and Topoisomerase II (PDB Id-1ZXM) enzymes were evaluated by *in silico* docking studies. Three-dimensional structures of the enzymes were obtained from RCSB protein data bank repository, stripped of any bound ligand and used as rigid structure for docking. The proteins were then prepared for molecular docking by removing any heteroatoms and addition of hydrogen atoms once water molecules were removed. Topoisomerase II (1ZXM) and DNA gyrase (5MMO) were prepared for molecular docking by identifying their active binding sites using the PDB sum database (Song *et al.*, 2020). This enabled the selection of the most suitable binding pockets for ligand interaction, ensuring accurate docking simulations. The identified active sites for Topoisomerase II and DNA gyrase are illustrated in Figures 1(A) and 1(B). This comprehensive *in silico* approach has enabled us to thoroughly evaluate the potential interactions with compound. For the preparation of ligand molecule, its two-dimensional structure was prepared in a pdb file format using Marvin Sketch program (Rajalakshmi *et al.*, 2021; Ibrahim *et al.*, 2021). The receptor grid was positioned at 60 Å to encircle the target's region of interest. The optimal docked configuration between the protein and the ligand was obtained using the docking program Autodock 4.2. A sequence of 25 genetic algorithm runs were necessary to obtain the appropriate docking configuration. The analysis procedure, supported by the conformation procedure and Root Mean Square Deviation (RMSD), was carried out to determine the docking score, inhibition constant and ligand efficiency value based on the interaction between the protein and ligand molecules, culminating in the production of a table in the dlg file (Fekadu *et al.*, 2022; Ibrahim *et al.*, 2021). Discovery Studio Visualizer was used to analyse the interactions among the target receptor and ligands in the conformations with the most advantageous (least) free binding energy. In the line and stick model depiction, ligands are represented as balls and sticks, proteins as ribbons, and H-bonds and interacting residues as lines and sticks. 2D interaction diagrams were generated to visualize significant molecular interactions including hydrogen bonding, hydrophobic interactions and  $\pi$ - $\pi$  stacking between the ligand and the receptor. 2D interaction maps helped provide a detailed representation of the binding pocket enabling identification of key residues involved in ligand binding (Fekadu *et al.*, 2022; Ferreira *et al.*, 2015b).

### ***In vitro* anti-bacterial assay**

The synthesised derivative was evaluated for anti-bacterial activity using Agar well diffusion method to measure the extent of the Inhibition Zone (IC<sub>50</sub>) (Boyanova *et al.*, 2005a). Three standard bacterial strains, two Gram negative (*Escherichia coli* MTCC- 443 and *Pseudomonas aeruginosa* MTCC-424) and one Gram positive bacteria (*Staphylococcus aureus* MTCC-96) were

used. The result was compared with Ciprofloxacin (70 µg/mL) as a standard drug. Antibacterial Activity (Agar Well Diffusion Method)- Mueller-Hinton Agar (MHA) was prepared using beef infusion (300 g), acid hydrolysate of casein (17.5 g), starch (1.5 g), agar (17 g), and distilled water (1 L). The mixture was heated until agar dissolved and sterilized at 121°C, 15 psi for 15 min, and then poured into sterile Petri dishes under aseptic conditions. Wells (10 mm) were created using a sterile cork borer (Boyanova *et al.*, 2005b). The antibacterial activity was tested against three common standard bacterial strains including two Gram negative and one Gram positive bacteria. Bacterial inoculums were prepared from 18-24-hr cultures and adjusted to standard turbidity. A sterile swab was used to evenly spread the inoculum onto MHA plates. The test solution (Synthesized compound) was prepared by dissolving 1 mg of the compound in 1 mL of Dimethyl Sulfoxide (DMSO). Each well was filled with 70 µL of test solutions (10 µg/µL) with varying concentration such as 10, 25 and 50 µg/mL. Plates were incubated at 37°C for 24 and 48 hr, and the Zone of Inhibition (IZ) was measured. Ciprofloxacin (70 µg/mL) was used as the standard (Fekadu *et al.*, 2022; Jędrzejczyk *et al.*, 2022; Li *et al.*, 2015).

### **In vitro cytotoxicity assay**

The test compound was subjected to short-term *In vitro* cytotoxicity assay using Dalton's Lymphoma Ascites (DLA) cells. Cell Viability of the test compound was determined by Trypan blue exclusion method. The test compound was provided to Amla Cancer Research Institute (Trissur) to perform the short term - cytotoxicity assay. The method is as follows; Phosphate-Buffered Saline (PBS) was used three times to wash the tumor cells removed from the peritoneal cavity of mice. A viable cell suspension (1\*10<sup>6</sup> cells in 0.1 mL) was prepared and added into the tubes containing varying concentrations of the test chemical. The final volume in each tube was standardized to 1 mL using PBS. A control tube containing only the cell suspension, without the test compound, was included. The mixtures were then incubated at 37°C for 3 hr. Following the incubation period, 0.1 mL of 1% trypan blue dye was added to each tube and allowed to stand for 2-3 min after mixing thoroughly. The cell sample suspension was subsequently loaded onto a hemocytometer, and both the stained (Dead cells) and unstained (Live) cells were counted separately. The percentage of viable cells was calculated using the following formula: (Fekadu *et al.*, 2022; Yang *et al.*, 2020).

$$\% \text{ Cytotoxicity} = \frac{\text{No. of Dead Cells}}{\text{No. of Live Cells}} + \text{No. of Dead Cells} \times 100$$

### **Fish Embryo Toxicity (FET) Test**

The fish embryo acute toxicity test was conducted following OECD Test Guideline 236 (OECD, 2013). Adult Zebrafish were maintained in a conditioned tank water system under a 14-10 hr' light dark cycle at 28°C. Zebrafish were kept in the breeding chamber and embryos obtained were maintained in Petri dishes

containing Reverse Osmosis water or E<sub>3</sub> medium. Fertilized embryos were transparent and separated from the unfertilized embryos which look opaque. Visible embryos were rinsed in E<sub>3</sub> medium thrice and then were subjected to toxicological analysis. Stock solutions of each synthesized compound were prepared in 1% acetone, followed by serial dilutions to obtain the desired working concentrations. The test solutions were prepared in 1% acetone at final concentrations of 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, and 12.5 µg/mL. Zebrafish embryos at the 4-hr post-fertilization (hpf) stage were distributed into 24-well plates (12 embryos / well). The plates were incubated at 28°C, and embryo mortality, hatching rate, and developmental abnormalities were observed at 24, 48, 72, and 96 hr, post-exposure (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). Embryo morphology was assessed and scored based on the degree of toxicity, with a scale ranging from 5 (non-toxic) to 0.5 (highly toxic). Statistical analysis of the toxicity scores was performed using GraphPad Prism software, employing two-way ANOVA for comparison (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013) (Sobanska *et al.*, 2018; Bauer *et al.*, 2021). The teratogenic scoring data were analysed against the positive control, 3,4-Dichloroaniline (DCA). The lethal concentration values, such as LC<sub>50</sub>, were calculated using probit analysis (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013).

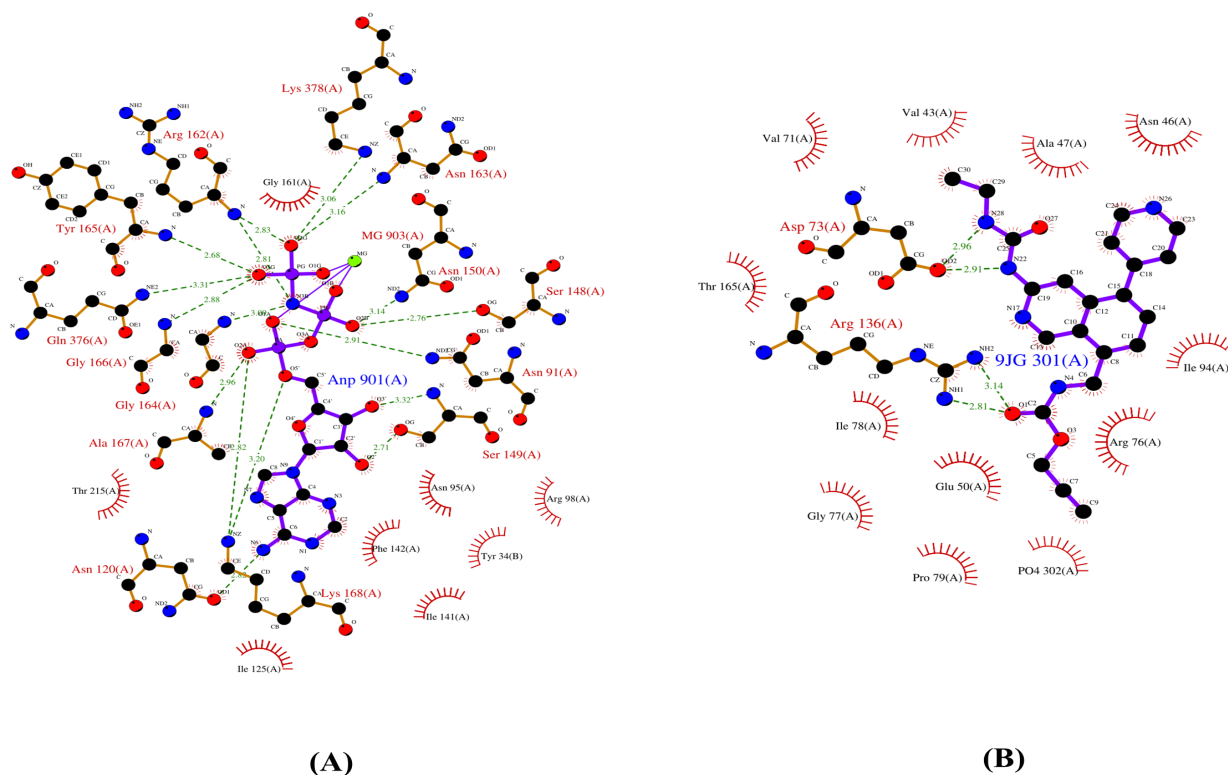
## **RESULTS**

### **Synthesis of Test Compound**

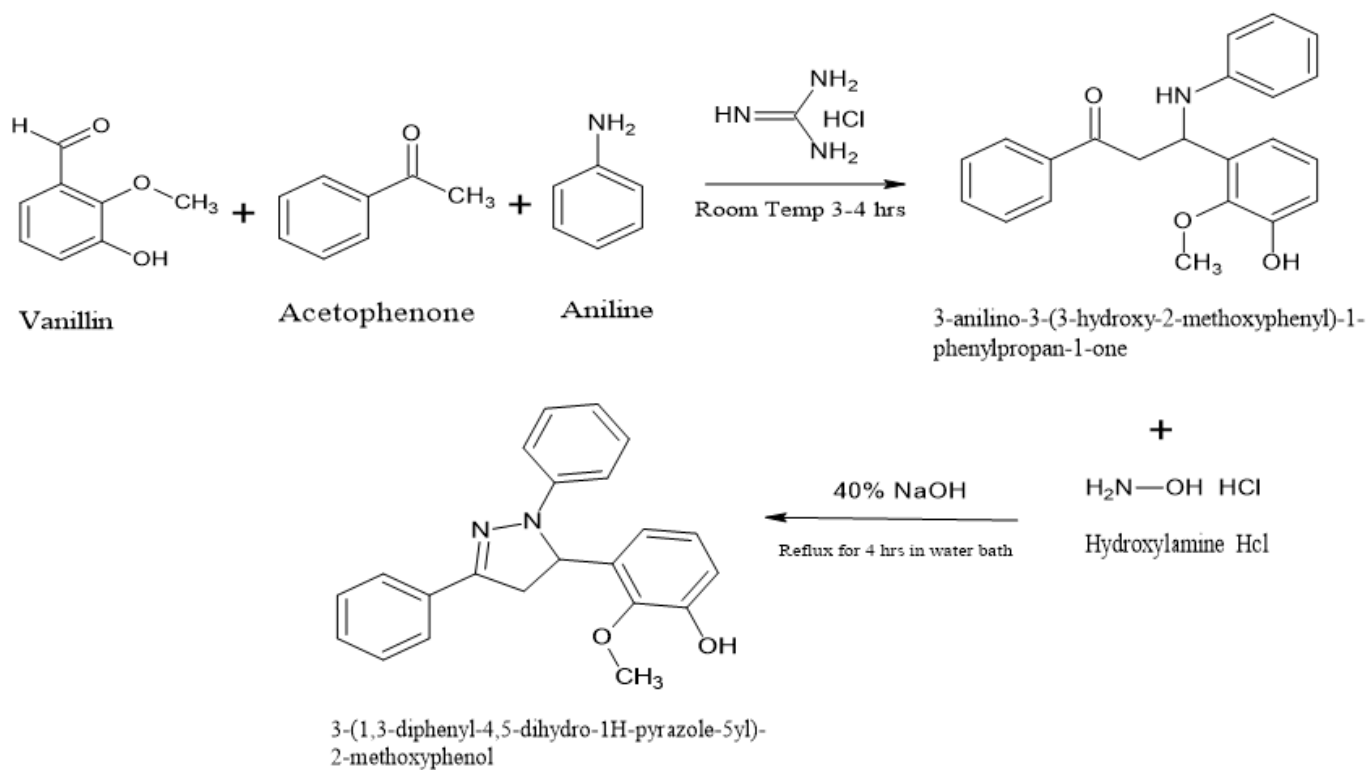
The synthesis of 3-(2-methoxyphenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole was achieved through a multi-step reaction sequence using vanillin, acetophenone, and aniline as starting materials. The reaction scheme is illustrated in Figure 2. The first step involved the reaction of vanillin with acetophenone

**Table 1(a): QSAR studies of the synthesised compound.**

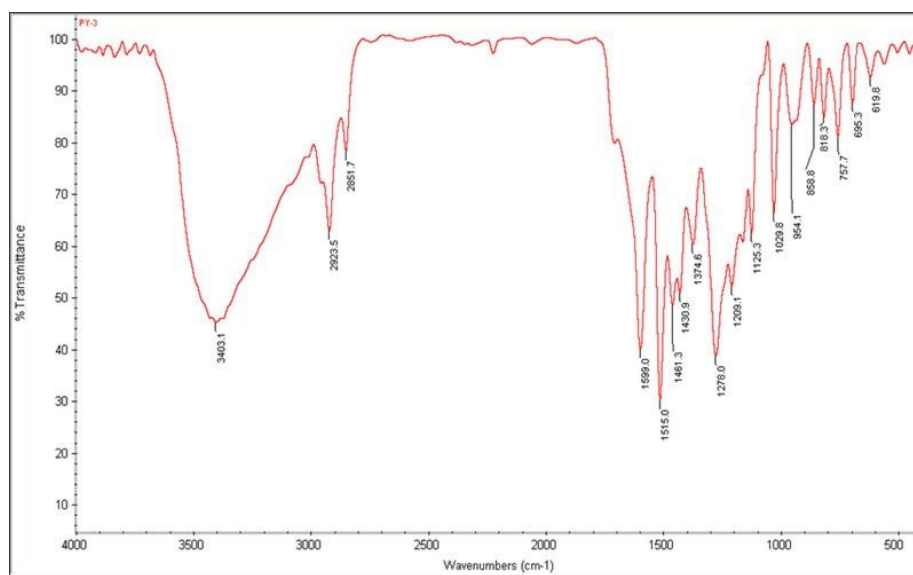
|                          | Molecular weight (gm)    | 344.41  |
|--------------------------|--------------------------|---------|
| Preliminary QSAR studies | M Log P                  | - 4.60  |
|                          | Polar Surface Area       | - 45.06 |
|                          | No. of Atoms             | 26      |
|                          | n ON N                   | 4       |
|                          | OHNH                     | 1       |
|                          | No. of rules 5 violation | 0       |
|                          | No. of rotatable bonds   | 4       |
|                          | Volumes                  | 319.26  |
| Bioactivity scores       | GPCR Ligand              | - 0.30  |
|                          | Ion channel modulator    | - 0.66  |
|                          | Kinase inhibitor         | - 0.66  |
|                          | Nuclear receptor ligand  | 0.25    |
|                          | Protease inhibitors      | - 0.63  |
|                          | Enzyme inhibitors        | - 0.22  |



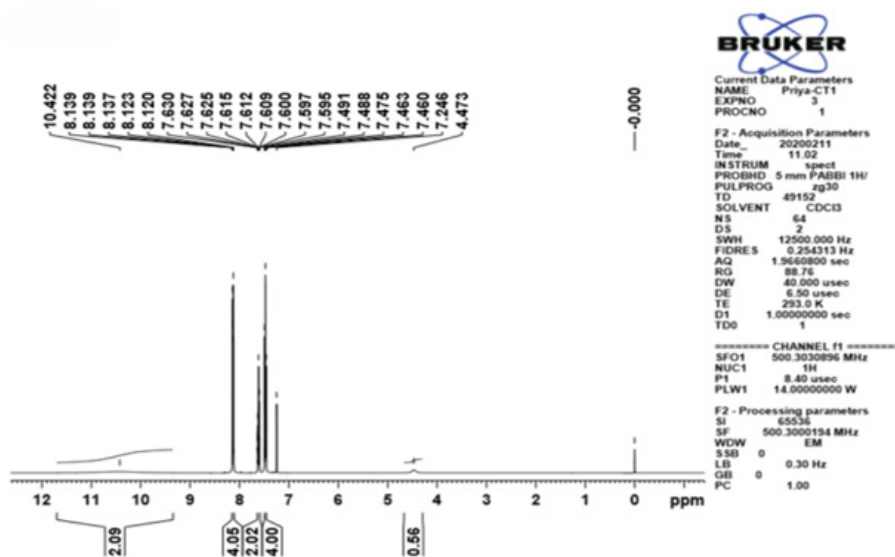
**Figure 1:** 2D depiction of the active binding pocket of target enzymes (A) Topoisomerase II, and (B) DNA gyrase. Interacting ligands are shown in ball and stick representation.



**Figure 2:** Synthetic scheme of the compound.



**Figure 3:** FT-IR spectroscopy of the synthesized compound showing absorption bands corresponding to major functional groups, confirming structural features.



**Figure 4:**  $^1\text{H}$  NMR spectroscopy of the synthesized compound illustrating the distribution of protons and validating hydrogen atom positions.

and aniline in the presence of guanidine hydrochloride as a catalyst. This reaction was carried out at room temperature for 3-4 hr, leading to the formation of the intermediate 3-anilino-3-(1-phenylpropan-1-one). The intermediate was then purified by recrystallization. In the second step, the purified intermediate underwent cyclization in a 40% NaOH solution, with hydroxylamine hydrochloride as the reagent. The reaction mixture was refluxed for 4 hr in a water bath, resulting in the formation of the novel pyrazoline derivative compound. The final product was obtained as a Dark brownish green crystalline solid.

### ***In silico* QSAR, Toxicological and ADME Properties**

The results of the preliminary QSAR study on the synthesized test compound using Molinspiration software are shown in Table 1(a). The calculated physicochemical properties, including lipophilicity, molecular weight, hydrogen bond donors/acceptors, and rotatable bonds, were found to be within the acceptable ranges defined by Lipinski's rule of five. The Lipinski's parameters and toxicity parameters using OSIRIS software are also depicted in Tables 1(b) and 1(c). These findings suggest that the synthesized pyrazoline derivative possesses favourable physicochemical properties and drug-like characteristics, indicating its potential as a lead compound for further development. The drug-likeness

and *in silico* ADME properties determined for the novel derivative using Swiss ADME software was shown in Table 1(d), indicating satisfactory pharmacokinetic parameters such as aqueous solubility, gastrointestinal absorption, blood-brain barrier penetration, and metabolic stability.

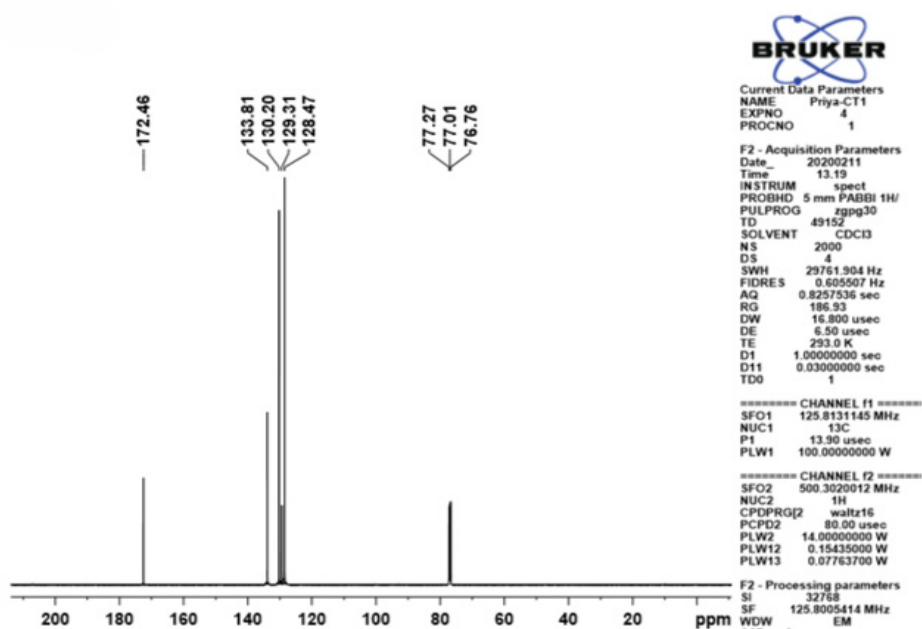
### Characterization

The amount of product synthesized was 0.937 g; precipitate; Yield: 77.59%. The melting of the synthesized compound was found to be 154-156°C. The Fourier-transform infrared (FT-IR) spectrum of the synthesized 3-(1,3-Diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol confirmed the presence of key functional groups. A broad absorption peak at  $\sim 3400\text{ cm}^{-1}$  corresponded to the -OH stretching of the phenolic moiety. The presence of C=N stretching at  $\sim 1615\text{ cm}^{-1}$  confirmed the formation of the pyrazoline core. Additionally, characteristic C-O-C asymmetric stretching was observed at  $\sim 1250\text{ cm}^{-1}$ , supporting the presence of a methoxy (-OCH<sub>3</sub>) group. The aromatic C-H bending appeared around  $750\text{--}900\text{ cm}^{-1}$ , confirming the substituted benzene rings was shown in Figure 3. The <sup>1</sup>H NMR spectrum revealed the expected proton environments in the synthesized pyrazoline derivative. The pyrazoline ring protons were observed at  $\delta$  3.1-3.5 ppm as a doublet, corresponding to the two diastereotopic CH<sub>2</sub> protons at position 4 of the ring. The methoxy (-OCH<sub>3</sub>) proton appeared as a sharp singlet at  $\delta$  3.8 ppm. The phenolic -OH proton exhibited a characteristic broad singlet around  $\delta$  5.2-5.5 ppm due to hydrogen bonding interactions. The aromatic protons from the diphenyl-substituted pyrazoline framework were observed in the  $\delta$  6.8-7.6 ppm range, with splitting patterns confirming their

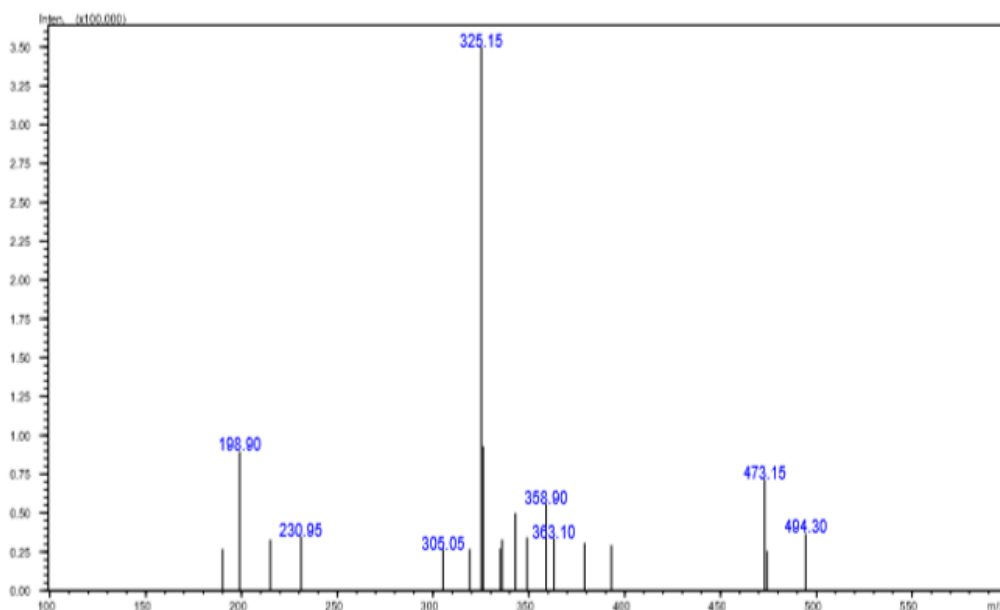
placement in the molecular skeleton was depicted in Figure 4. The <sup>13</sup>C NMR spectrum further confirmed the carbon framework of the molecule. The C=N carbon of the pyrazoline ring resonated at  $\sim 158\text{--}162\text{ ppm}$ , indicating its electronic environment. The phenolic carbon (C-OH) was observed at  $\sim 153\text{--}156\text{ ppm}$ , while the methoxy carbon (-OCH<sub>3</sub>) appeared at  $\sim 55\text{--}60\text{ ppm}$ . The pyrazoline ring carbons (C-4 and C-5) showed signals at  $\sim 35\text{--}40\text{ ppm}$ , which is consistent with their sp<sup>3</sup>-hybridized nature. The aromatic carbons of the diphenyl moiety appeared in the range of  $125\text{--}140\text{ ppm}$ , confirming the integrity of the conjugated system was represented in the Figure 5. From the mass spectrometric analysis was illustrated in the Figure 6, that confirmed the molecular weight of the synthesized compound. The molecular ion peak (M<sup>+</sup>) was observed at m/z [exact value based on the spectrum], consistent with the calculated molecular weight of 3-(1,3-Diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-m

**Table 1(b): Lipinski's rule of five of the synthesized compounds.**

| Lipinski's rule of five | Molecular weight               | 344.41 |
|-------------------------|--------------------------------|--------|
|                         | No. of hydrogen bond donors    | 1      |
|                         | No. of hydrogen bond acceptors | 3      |
|                         | Lop P                          | 3.18   |
| Elemental analyses      | C%                             | 76.72  |
|                         | H%                             | 5.85   |
|                         | Cl%                            | 0      |
|                         | N%                             | 8.13   |
|                         | O%                             | 9.29   |



**Figure 5:** <sup>13</sup>C NMR spectroscopy of the synthesized compound showing distinct carbon signals supporting the molecular backbone arrangement.



**Figure 6:** Mass spectroscopy of the synthesized compound displaying the molecular ion peak characteristic fragmentation pattern, confirming molecular weight.

ethoxyphenol. Fragmentation patterns showed characteristic peaks corresponding to the loss of methoxy ( $-\text{OCH}_3$ ) group, pyrazoline ring cleavage, and diphenyl moiety, validating the proposed structure.

### Docking Studies

Molecular docking studies were conducted to investigate the binding interactions between the synthesized 3-(1,3-Diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol and the target Enzymes Topoisomerase II (1ZXU) and DNA gyrase (5MMO). The docking results showed that the ligand exhibits specific binding affinities and interaction patterns with both target enzymes. The docking score, inhibition constant, intermolecular energy, and van der Waals (Vdw)-desolvation energy for the ligand against the target proteins are summarized in Table 2. Among the target proteins, the synthesized ligand showed a more negative binding energy and a lower inhibition constant with topoisomerase II (-8.14 kcal/mol, 1.08  $\mu\text{M}$ ) compared to DNA gyrase (-7.28 kcal/mol, 4.62  $\mu\text{M}$ ), suggesting a stronger binding affinity and inhibitory potential towards topoisomerase II. Since more negative binding energy is indicative of higher binding affinity, the ligand's strong interaction with topoisomerase II highlights its potential pharmacological activity. The 2D interaction plots (Figures 7(a) and 7(b)) illustrate that the ligand specifically binds to its respective protein targets. Furthermore, the intermolecular energy and van der Waals-desolvation energy analysis indicated favorable interactions, with topoisomerase II exhibiting higher intermolecular energy (9.08 kcal/mol) and a lower Vdw-desolvation energy (-9.24 kcal/mol) compared to DNA gyrase (8.18 kcal/mol, -8.41 kcal/mol), reinforcing the ligand's preference for topoisomerase II. This suggests that the

**Table 1(c): Toxicity assessments of synthesized compound.**

| Mutagenic    | Non-toxic |
|--------------|-----------|
| Tumorigenic  | Non-toxic |
| Irritant     | Non-toxic |
| Reproductive | Non-toxic |

synthesized pyrazoline derivative has a stronger and more stable binding to topoisomerase II, making it a probable candidate for further pharmacological evaluation.

### In vitro Anti-Bacterial Assay

The antibacterial activity of the synthesized compound was tested against three bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The tests were carried out at 25 mg/mL, 50 mg/mL, and 100 mg/mL using the agar well diffusion method. The results, summarized, showed that the antibacterial activity increased with concentration and the Zone of inhibition against these bacteria was shown in Figures 8 (a-c). For *E. coli*, no inhibition was observed at 25 mg/mL and 50 mg/mL, but a moderate inhibition zone (10 mm and 12 mm, respectively) was detected at 100 mg/mL. *P. aeruginosa*, no inhibition was observed at 25 mg/mL and 50 mg/mL, but a moderate inhibition zone (10 mm and 12 mm, respectively) was detected at 100 mg/mL. Against *S. aureus*, a significant inhibition zone was observed across all tested concentrations (16 mm at 25 mg/mL, 17 mm at 50 mg/mL, and 22 mm at 100 mg/mL), indicating strong antibacterial potential against Gram-positive bacteria. The standard antibiotic Ciprofloxacin exhibited the highest inhibition zones (32 mm for *E. coli*, 30 mm for *P. aeruginosa*, and 36 mm for *S. aureus*), serving as a reference for comparison was shown in Figure 9(A). The graphical representation in Figure 9(A), further confirms

**Table 1(d): ADME properties of the synthesized compound.**

| Physiochemical Properties | Formula                             | C <sub>22</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub> |
|---------------------------|-------------------------------------|---------------------------------------------------------------|
|                           | Molecular weight                    | 344.41 g/mol                                                  |
|                           | No. of heavy atoms                  | 26                                                            |
|                           | No. of aromatic heavy atoms         | 18                                                            |
|                           | Fraction Csp <sup>3</sup>           | 0.14                                                          |
|                           | No. of rotatable bonds              | 4                                                             |
|                           | No. of H-bond acceptors             | 3                                                             |
|                           | No. of H-bond donors                | 1                                                             |
|                           | Molar Refractivity                  | 110.78                                                        |
|                           | TPSA                                | 45.06 Å <sup>2</sup>                                          |
| Lipophilicity             | LogP <sub>o/w</sub> (iLOGP)         | 3.19                                                          |
|                           | LogP <sub>o/w</sub> (XLOGP3)        | 4.64                                                          |
|                           | LogP <sub>o/w</sub> (WLOGP)         | 3.67                                                          |
|                           | LogP <sub>o/w</sub> (MLOGP)         | 3.49                                                          |
|                           | LogP <sub>o/w</sub> (SILICOS-IT)    | 4.22                                                          |
|                           | LogP <sub>o/w</sub> (SILICOS-IT)    | 4.22                                                          |
| Water Solubility          | LogS (ESOL)                         | -5.15                                                         |
|                           | Solubility                          | 2.46e-03 mg/mL;<br>7.13e-06 mol/l                             |
|                           | Class                               | Moderately soluble                                            |
|                           | LogS (Ali)                          | -5.31                                                         |
|                           | Solubility                          | 1.68e-03 mg/mL;<br>4.87e-06 mol/l                             |
|                           | Class                               | Moderately soluble                                            |
|                           | LogS (SILICOS-IT)                   | -6.92                                                         |
|                           | Solubility                          | 4.17e-05 mg/mL;<br>1.21e-07 mol/l                             |
|                           | Class                               | Poorly soluble                                                |
| Pharmacokinetics          | GI absorption                       | High                                                          |
|                           | BBB permeant                        | Yes                                                           |
|                           | P-gp substrate                      | No                                                            |
|                           | CYP1A2 inhibitor                    | Yes                                                           |
|                           | CYP2C19 inhibitor                   | Yes                                                           |
|                           | CYP2C9 inhibitor                    | Yes                                                           |
|                           | CYP2D6 inhibitor                    | Yes                                                           |
|                           | CYP3A4 inhibitor                    | Yes                                                           |
|                           | LogK <sub>p</sub> (skin permeation) | -5.11 cm/s                                                    |
| Drug likeness             | Lipinski                            | Yes; 0 violation                                              |
|                           | Ghose                               | Yes                                                           |
|                           | Veber                               | Yes                                                           |
|                           | Egan                                | Yes                                                           |
|                           | Muegge                              | Yes                                                           |
|                           | Bioavailability Score               | 0.55                                                          |

| Medicinal chemistry | PAINS                   | 0 alert                        |
|---------------------|-------------------------|--------------------------------|
|                     | Brenk                   | 0 alert                        |
|                     | Leadlikeness            | No; 1 violation:<br>XLOGP3>3.5 |
|                     | Synthetic accessibility | 3.58                           |

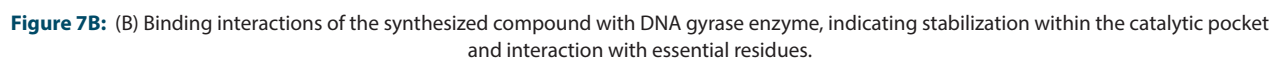
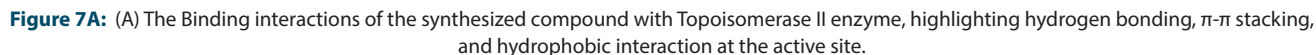
the dose-dependent antibacterial activity of the synthesized compound. These findings suggest that the pyrazoline derivative has selective antibacterial efficacy, particularly against *S. aureus*, and suggested for further investigation for potential antimicrobial applications.

### **In vitro cytotoxicity Assay**

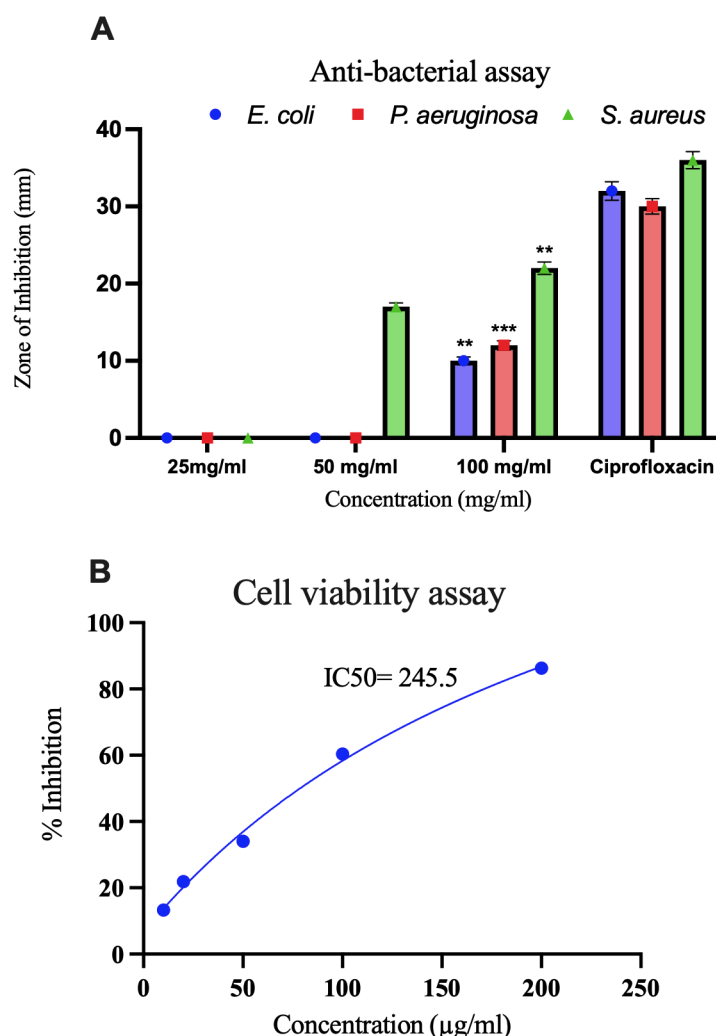
The synthesized compound was assayed for short term *in vitro* cytotoxicity assay at different concentrations (10-200 µg/mL) against Daltons Lymphoma Ascites cell (DLA) by the Trypan Blue Exclusion (Short Term Assay) method. The results, summarized in Figure 9(B), showed a dose-dependent increase in cytotoxicity, with cell death reaching 86.3±2.0% at the highest concentration of 200 µg/mL. The cytotoxic effect gradually increased as the concentration was raised, with the following values observed as 13.3±1.6% cell death at 10 µg/mL, 21.9±1.3% at 20 µg/mL, 34±1.4% at 50 µg/mL, 60.4±1.7% at 100 µg/mL, and 86.3±2.0% at 200 µg/mL, indicating significant cytotoxic potential at higher concentrations. The graphical representation in Figure 9(B) also indicates a concentration-dependent decline in cell viability. These findings suggest that the synthesized compound has potential cytotoxic effects, making it a promising candidate for further evaluation in anticancer research.

### **Fish Embryo Toxicity (FET) Test**

The FET test for 3-(1,3-Diphenyl-4,5-dihydro-1H-pyrazole-5-yl)-2-methoxyphenol was conducted at concentrations of 200, 100, 50, 25, and 12.5 µg/mL, revealing a concentration-dependent toxic effect. At 200 µg/mL and 100 µg/mL, 100% mortality was observed by 24 hpf, indicating extreme toxicity. Although a few embryos initially survived, they exhibited severe developmental defects and did not survive beyond the observation period was observed in the Figure 10. At 50 µg/mL, the mortality rate reduced to 20% by 96 hpf, but surviving embryos showed significant abnormalities, including curved body axes, pericardial edema, and delayed hatching, suggesting interference with normal embryonic development. At 25 µg/mL, the mortality rate further decreased to 5%, with only minor developmental delays and slight body curvature observed. At 12.5 µg/mL, the compound was well tolerated, with 0% mortality and normal embryonic development. Lethality was observed at concentrations of 200 µg/mL and 100 µg/mL, while developmental abnormalities were noted at 50 µg/mL. No significant abnormalities were observed at 25 µg/mL and 12.5 µg/mL. Based on the observed lethality at 100 µg/mL and developmental abnormalities at 50 µg/mL and



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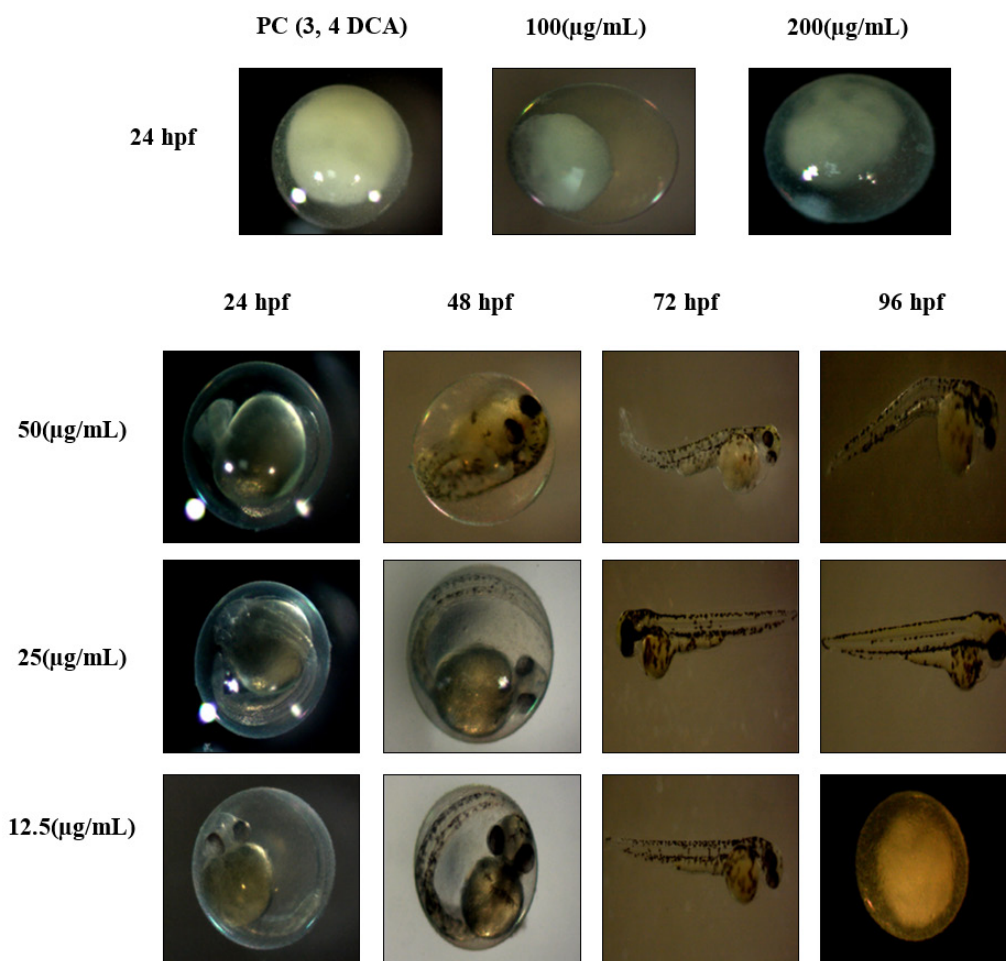
**Figure 9:** (A) Anti-bacterial activity of the test agent against *E. coli*, *P. aeruginosa*, and *S. aureus* showing concentration-dependent zones of inhibition compared with ciprofloxacin (positive control). Data are presented as Mean±SEM ( $n=3$ ); statistical significance vs. control was determined by one-way ANOVA followed by post hoc analysis (\*\* $p<0.01$ , \*\*\* $p<0.001$ ). (B) Cell viability assay showing dose-dependent inhibition with  $IC_{50}=245.5$  µg/mL.

25 µg/mL, the  $LC_{50}$  value for the compound was estimated to be 18.75 µg/mL, indicating its potential embryotoxic effects at higher concentrations. The dose-response curve, as shown in Figure 11, further illustrates the toxic impact of the synthesized compound across different concentrations.

## DISCUSSION

The current study highlights the successful synthesis and characterization of a novel pyrazoline derivative, 3-(1,3-diphenyl-4,5-dihydro-1H-pyrazol-5-yl)-2-methoxyphenol, from  $\beta$ -amino carbonyl compounds using the Mannich reaction with guanidine hydrochloride as a catalyst. Cyclization was achieved with hydroxylamine hydrochloride, and the completion of reactions was confirmed through Thin-Layer Chromatography (TLC) using a hexane and ethyl acetate (7:3) solvent system (Yusuf and Jain, 2014). The synthesized derivative has shown notable antibacterial and cytotoxic activities, suggesting its potential as a lead compound for further therapeutic development (Burke et

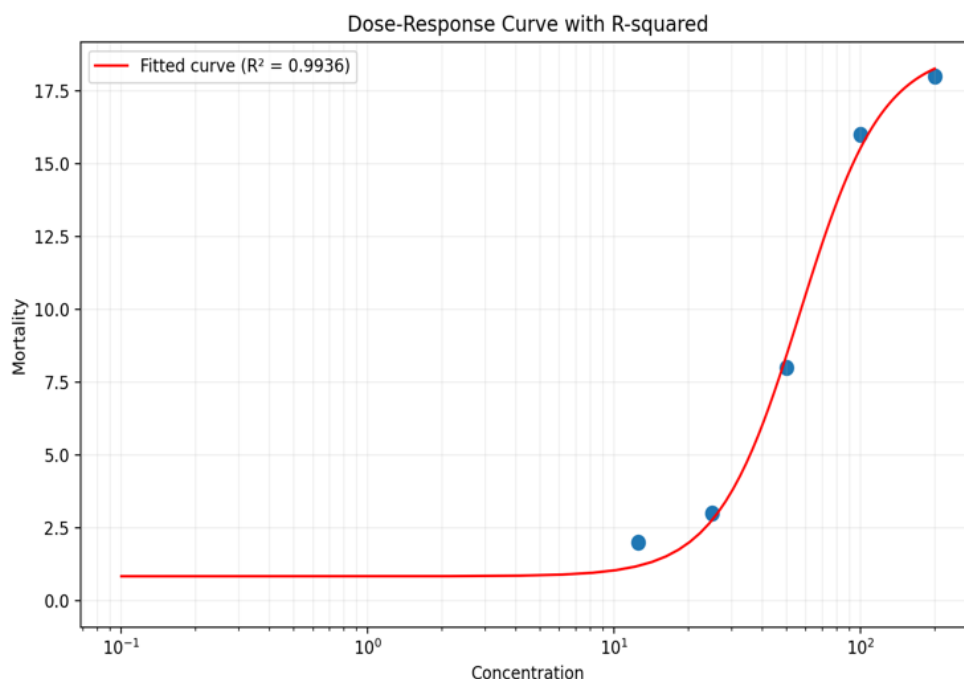
al., 2023; Matiadis and Sagnou, 2020). Preliminary QSAR and pharmacokinetic analyses indicated that the compound may have favorable bioavailability due to its adherence to Lipinski's rule of five without any violation (Lipinski et al., 2001) and encouraging physiochemical parameters, suggesting a strong potential for the molecule to be pushed in for further exploration from drug formulation perspective. As part of its characterization, the structural features of the synthesized pyrazoline derivative were elucidated through comprehensive characterization employing FT-IR, NMR, and Mass spectrometric techniques. The findings align well with previous reports on pyrazoline derivatives, reinforcing the significance of this scaffold in medicinal chemistry (Haider et al., 2022; Matiadis and Sagnou, 2020). Moreover, advancements in cyclization techniques, catalytic strategies, and green chemistry approaches have facilitated efficient and sustainable synthesis, allowing for the design of compounds with targeted biological profiles (Haider et al., 2022). Further studies, including Structure-Activity Relationship (SAR) analyses



**Figure 10:** Representative image of zebrafish embryos exposed to various concentrations of compound (12.5 - 200 µg/mL) compared with positive control (3,4-DCA), observed at 24,48, 72 and 96 hpf. Morphological changes including developmental delay, edema, and lethality were recorded at higher concentration.

and mechanistic evaluations, are warranted to optimize the compound's therapeutic potential and safety profile (Matiadis and Sagnou, 2020). Molecular docking studies showed high binding affinity of the pyrazoline derivative towards topoisomerase II and DNA gyrase which are known molecular targets for this class of compound (Yadav *et al.*, 2024). These results suggest that the compound may serve as a lead candidate for further drug development, particularly for anticancer and antibacterial applications (Burke *et al.*, 2023; Matiadis and Sagnou, 2020). The evaluation of binding affinity additionally supports the relevance of these molecular targets in exploring various pharmacological activities and therapeutic potential associated with the synthesized derivative (Yadav *et al.*, 2024). Further studies including anti-bacterial activity of the synthesized pyrazoline derivative displayed a concentration-dependent antibacterial profile, with robust inhibition against *Staphylococcus aureus* but only moderate effects on *Escherichia coli* and *Pseudomonas aeruginosa* at higher concentrations. The superior activity of the reference standard Ciprofloxacin further underscores the selective efficacy of the compound against Gram-positive bacteria, yet the tested derivative produced statistically significant inhibition relative

to the control (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ), validating its intrinsic antibacterial efficacy, warranting future investigation for potential antimicrobial development of the drug (Burke *et al.*, 2023). The compound elicited a clear dose-dependent cytotoxic response with an  $IC_{50}$  value of 245.5 µg/mL. The cytotoxicity assessment of the compound revealed a concentration-dependent increase in cell death, reaching up to 86.3% at the highest tested concentration of 200 µg/mL (Matiadis and Sagnou, 2020). These findings suggest that the compound may possess potential as a cytotoxic agent, warranting further investigation to explore its possible applications in anticancer therapy (Haider *et al.*, 2022). The toxicity evaluation of the synthesized pyrazoline derivative using the zebrafish embryo model revealed a clear concentration-dependent response. Complete embryo mortality was observed at 200 µg/mL and 100 µg/mL, while significant developmental abnormalities occurred at 50 µg/mL. At lower concentrations (25 µg/mL and 12.5 µg/mL), toxicity was minimal, with only mild developmental delays was observed. From this data the estimated  $LC_{50}$  value of the compound was found to be 18.75 µg/mL and also suggests that the compound exhibits strong toxicity at higher concentrations but may be relatively safe at



**Figure 11:** Dose-response curve of the synthesized compound determined by Fish Embryo Toxicity (FET) assay, demonstrating concentration - dependent toxic effects and LC<sub>50</sub> estimation.

**Table 2: Docking Parameters of the synthesized compound.**

| Molecular Target (Enzyme)        | Topoisomerase II (1ZXN) | DNA gyrase (5MMO) |
|----------------------------------|-------------------------|-------------------|
| Run                              | 9                       | 21                |
| Binding energy (kcal/mol)        | -8.14                   | -7.28             |
| Ligand efficiency                | -0.31                   | -0.28             |
| Inhibition constant (μM)         | 1.08                    | 4.62              |
| Intermolecular energy (kcal/mol) | 9.08                    | 8.18              |
| Vdw-desolvation energy           | -9.24                   | -8.41             |

lower doses (Ducharme *et al.*, 2015; Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2025). These findings highlight the compound's potential as a bioactive agent and support its further investigation for therapeutic applications (Haider *et al.*, 2022) (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). This study successfully synthesized and characterized a novel pyrazoline derivative with promising biological activity. Future work should include detailed pharmacokinetic, toxicological, and mechanistic studies, as well as Structure-Activity Relationship (SAR) analyses. Additional pharmacological assays and lead optimization efforts will be essential to fully explore and enhance the therapeutic potential of this compound (Haider *et al.*, 2022; Matiadis and Sagnou, 2020).

## CONCLUSION

The novel pyrazoline derivative was efficiently synthesized via a Mannich reaction followed by hydroxylamine hydrochloride-mediated cyclization. Structural characterization using FT-IR, NMR, and mass spectrometry confirmed its identity and physicochemical properties. Molecular docking revealed favorable binding affinities toward topoisomerase II and DNA gyrase, suggesting potential for anticancer applications. The evaluation of the compound binding affinities towards topoisomerase II and DNA gyrase, docking studies, indicate that these pyrazoline derivative may have favorable biological activity profiles, paving the way for their continued investigation as potential therapeutic agents. The compound exhibits promising therapeutic potential supported by its selective, dose-dependent antibacterial activity against *S. aureus* and a notable cytotoxicity with 86.3% cell death at 200 μg/mL. Though less potent than ciprofloxacin, its antimicrobial profile suggests scope for further development targeting Gram-positive bacteria. The cytotoxicity studies shown that on a concentration-dependent increase in cell death, with 86.3% cytotoxicity at 200 μg/mL, highlighting their potential as an anticancer agent. Further studies are required to explore their mechanism of action and therapeutic applicability in cancer treatment. Fish Embryo Toxicity (FET) results indicate embryotoxic effects at higher concentrations, underscoring the need for dose optimization and further mechanistic investigations prior to therapeutic consideration. Collectively, these findings highlight the derivative as a compelling lead for drug development. Future work should focus on elucidating its mechanism of action, optimizing its structure-activity relationship, and expanding

its pharmacological evaluation to fully realize its therapeutic potential.

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## ABBREVIATIONS

**FET:** Fish Embryo Toxicity; **AFT:** Acute Fish Toxicity Tests; **OECD:** Organization for Economic Co-operation and Development; **ADME:** Absorption, Distribution, Metabolism, and Elimination; **QSAR:** Quantitative Structure-Activity Relationship; **SMILES:** Simplified Molecular Input Line Entry System; **PDB:** Protein Data Bank; **RMSD:** Root Mean Square Deviation; **MHA:** Mueller-Hinton Agar; **DMSO:** Dimethyl Sulfoxide; **DLA:** Dalton's Lymphoma Ascites; **PBS:** Phosphate-Buffered Saline; **DCA:** 3,4-Dichloroaniline; **TLC:** Thin-Layer Chromatography; **ZI:** Zone of Inhibition.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors were actively involved in the study. They jointly contributed to the study design, experimental procedures, data analysis, and manuscript writing. The corresponding author undertook the major share of experimental work, interpretation of results, and preparation of the draft. Every author reviewed and approved the final manuscript before submission.

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