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New *N*-amino-5-cyano-6-pyridones as antimicrobial small molecules endowed with DNA gyrase a inhibitory activity: design, one-pot synthesis, biological assessment and *in silico* insights

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Abstract

A set of innovative *N*-amino-5-cyano-6-pyridones derivatives was developed and produced using one-pot three-component procedures. The evaluated molecules were examined for their antimicrobial efficacy. Based on the acquired findings, most of the investigated compounds had promising antimicrobial properties. Out of these derivatives of 3-cyanopyridine, compounds **3d** and **3e** exhibited minimum inhibitory concentrations (MIC) of 3.91 µg/mL against *E.coli*. In vitro evaluation of DNA gyrase A displayed that molecule **3d** exhibited promising potency as an inhibitor, with an IC_{50} value of 1.68 µg/mL compared to ciprofloxacin (IC_{50} = 0.45 µg/mL). Furthermore, it was observed that molecule **3e** exhibited a moderate inhibitory effect, as indicated by its IC_{50} value of 3.77 µg/mL. A kinetics study conducted to assess the time required to kill *E. coli* bacteria demonstrated that gentamycin and compounds **3d** and **3e** exhibited bactericidal effects within a time frame of 90–120 min. Based on the ADME predictions, compounds **3d** and **3e** are expected to have favorable oral bioavailability and are unlikely to penetrate the blood-brain barrier. Computational mutagenicity and tumorigenicity studies were conducted on compounds **3d** and **3e**. The molecular docking investigation has conclusively demonstrated the binding of compounds **3d** and **3e** to the target DNA gyrase A enzyme, further reinforcing the existing data.

Keywords One-pot synthesis, Antimicrobial, Pyridones, DNA gyrase

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Introduction

In developing countries, there is a high incidence of microbial infections, which are leading to a rising number of illnesses and deaths. The global rise of antibacterial resistance poses an increasingly significant danger to human health [1]. The pace of finding new antibacterials is significantly surpassed by the speed at which resistance is growing, and there is a pressing demand for the creation of novel medicines for antibacterial purposes [2, 3]. Developing countries are particularly worried about the over-abuse of antibiotics in human as well as animal communities. Consequently, these circumstances result in the physician having fewer treatment options, longer hospital stays, higher costs, greater vulnerability to infection, and elevated mortality rates [4]. Antibiotic resistance poses a significant risk to the successful prevention and treatment of a growing range of bacterial diseases, rendering it one of the most prominent obstacles to worldwide medical systems. Presently, an estimated 700,000 individuals globally succumb to drug-resistant diseases annually [5]. N-substituted 2-pyridones are nitrogen-containing heteroarenes that have antimicrobial properties, rendering them appropriate for several medical uses [6]. The FDA database contains over 50 drugs derived from pyridine, such as pyridostigmine for myasthenia gravis, omeprazole for peptic ulcer, lornoxicam for arthritis, amlodipine for hypertension, milrinone for cardiac support, torsemide as a diuretic, bromazepam for anxiety, tacrine for Alzheimer's disease, abemaciclib for breast cancer, and omidenepag for glaucoma [7, 8]. Cyanopyridine derivatives are crucial intermediates in the synthesis of biologically active compounds, thus they play an important role in chemical synthesis [9]. Pharmaceuticals greatly benefit from the utilization of compounds that incorporate the pyridine-3-carbonitrile group [10]. Figure 1 displays the effectiveness of pyridine/Pyridone as an antimicrobial agent. Specifically, 6-dimethyl-2-oxo-pyridine-3-carbonitrile **I** demonstrated an antibacterial effect against *Escherichia coli* (IZ=22.8 mm) and *Pseudomonas aeruginosa* (IZ=21.4. mm) close to that of the reference streptomycin (IZ=25 mm against *E. coli* and 24 mm against *P. aeruginosa*) [11]. Furthermore, it was discovered that the compound **II**, a derivative of 1,4-dihydropyridine, exhibited significantly higher activity (IZ=25 mm) in comparison to the conventional ciprofloxacin (IZ=22 mm) against *S. aureus* [12]. Moreover, the presence of the pyridine ring in molecule **III** played a crucial role in its effectiveness towards *C. albicans*. The minimum inhibitory concentration (MIC) result for this molecule **III** was 15.6 mg/mL [13]. Moreover, pyridine-3-carbonitrile compound **IV** demonstrated a comparable inhibitory activity towards *E. coli* (MIC=0.013 µM) to the reference medication amoxicillin (MIC=0.01 µM) [14]. Moreover, molecule **V** had remarkable efficacy towards gram-negative bacteria, specifically *E. Coli*, with an IZ measuring 22 mm, while the reference ofloxacin showed IZ value of 24 mm [15]. The pyridone derivative **VI** showed significant antibacterial activity toward *B. subtilis*, and *S. aureus* (MIC=0.078 and 0.0024 mg/mL, respectively) compared to cefaclor (MIC=0.0024 mg/mL) [16]. Further, Compound **VII** exhibited a wide range of antibacterial activity (MIC of 1.95 µg/mL for *Streptococcus pneumonia*, 0.98 µg/mL for *B. subtilis*, and 1.9 µg/mL for *Salmonella typhimurium*) that was equipotent to the reference drugs ampicillin (against *Streptococcus pneumonia* and *B. subtilis)* and gentamycin (against *Salmonella typhimurium*). Furthermore, it demonstrated antifungal activity towards *Aspergillus fumigatus* and *C. albicans* (MIC values of 1.95 µg/mL and 7.81 µg/mL, respectively) relative to the antifungal reference amphotericin B (MIC=1.95 µg/mL) [17]. Compound **VIII** demonstrated potent antibacterial effects against methicillin-resistant *S. aureus* equal to that of vancomycin (MIC=1 µg/mL) [18]. In addition, compound **IX** exhibited strong antifungal activity equivalent to that of nystatin against *C. albicans* (MIC=1.95 µg/mL) [19]. Furthermore, 2-pyridones are important class of antimicrobial compounds because of their efficacy against the bacterial type II DNA topoisomerases (DNA gyrase and topoisomerase IV). Also, 2-oxopyridine derivatives are isomeric to 4-oxopyridine core, which is present in the fluoroquinolone class of antibiotics [20]. From the reported DNA gyrase inhibitors, compounds **X** and **XI** displayed good anti-gyrase activity (IC_{50}=21.97 and 39.15 µM, respectively) compared to the reference ciprofloxacin (IC_{50}=26.32 µM) [20]. As well, the 3-cyanopyridine derivative **XII** (IC_{50}=0.44 µM) showed nearly equal activity against DNA gyrase to ciprofloxacin (IC_{50}=0.42 µM), while compound **XIII** exhibited half the activity of ciprofloxacin (IC_{50}=0.94 µM) [21]. Furthermore, compound **XIV** containing fused pyridine core demonstrated DNA gyrase inhibitory activity (IC_{50} value of 7.35 µM), which was more potent than Ciprofloxacin (IC_{50} value of 47.68 µM) [22].

Our research focused on synthesizing novel nicotinonitriles to explore their antibacterial properties. We assessed their antimicrobial activity by determining their IZ (inhibition zone) and MICs (minimum inhibitory concentrations). The potential compounds that have demonstrated strong antibacterial effects will undergo additional investigation using in vitro enzyme potency assessment for DNA gyrases A, in order to examine their antibacterial properties. Additionally, a bacterial killing kinetic assay was performed to evaluate the antibacterial activity of the most active compounds over time and to predict their safety profile through assessing their cytotoxic effect on normal cells using the MTT technique. The study will involve conducting docking analyses to investigate the binding affinities of the enzyme of

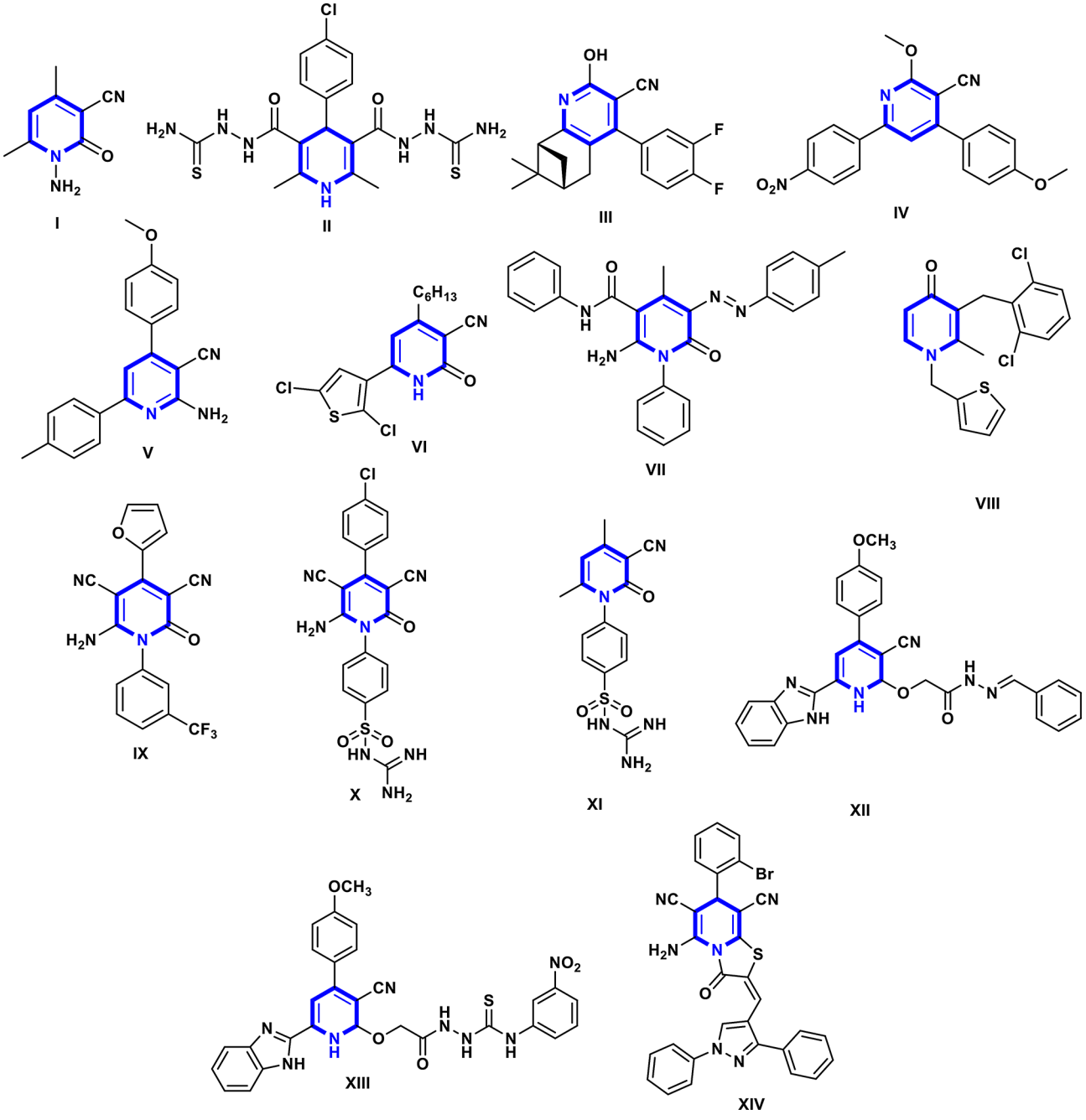


Fig. 1 Reported pyridine/Pyridone as antimicrobial compounds

interest, as well as performing in silico ADME forecasting to assess the physicochemical properties.

Rationale and design

The optimization approaches for the compound **I** depend on the strategies shown in Fig. 2. Compound **I** exhibited significant antibacterial properties towards *Pseudomonas aeruginosa* and *E. coli* with IZ 21.4, and 22 mm, respectively [11]. The objective of this work was to substitute the methyl group at position 4 with aryl moiety, explore the impact of different substituents, and replace the methyl group at position 6 of compound **I** with a hydrophilic amino group at position 2, which could potentially participate in hydrogen bonding. Furthermore, our objective was to preserve the *N*-aminocynopyridone ring. Carboxamide or carbothiomide, which may also be significant in the formation of hydrogen bonds, is additionally introduced.

As the *N*-aminocynopyridone ring is considered the pharmacophoric moiety responsible for the stated

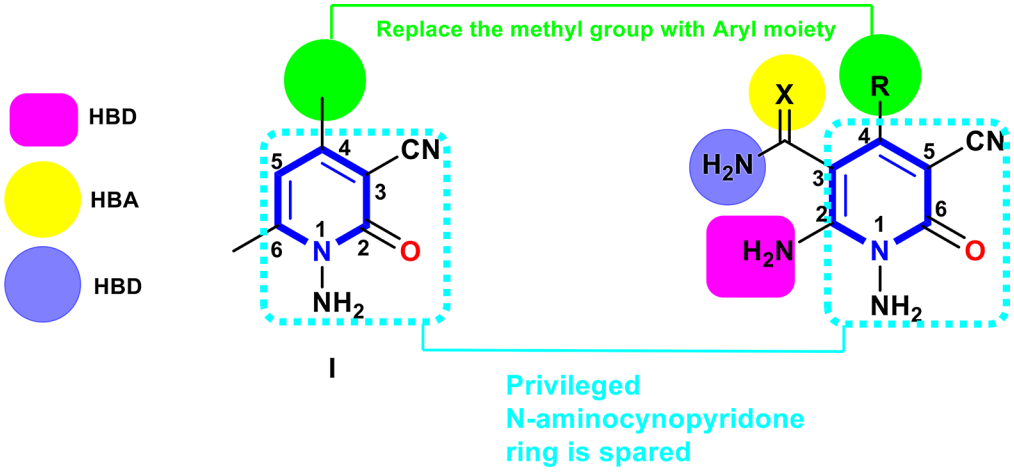


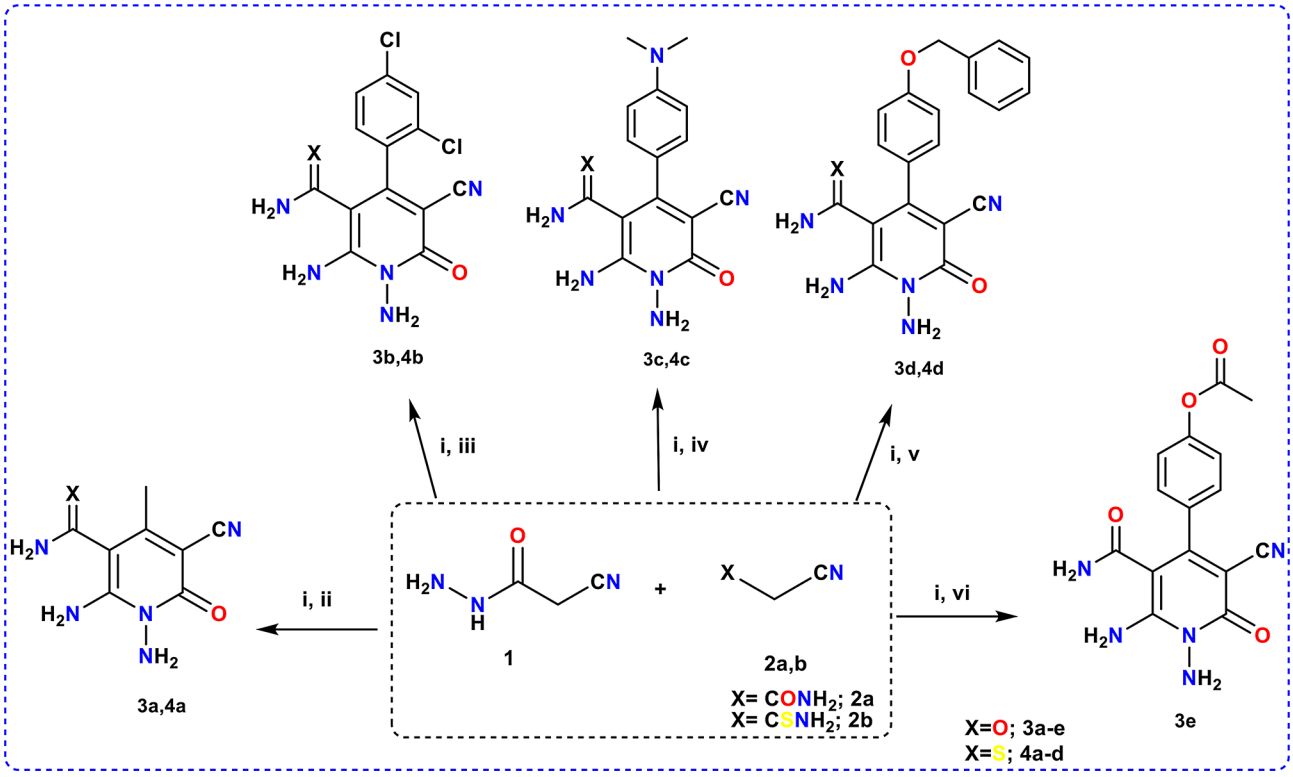
Fig. 2 The design of new cyanopyridones

activity, our research focused on synthesizing a variety of new derivatives. Specifically, we aimed to develop derivatives based on 6-oxo-1,6-dihydropyridine-3-carboxamide/carbothioamide. We will evaluate the antimicrobial properties of all compounds against two strains of fungi, two strains of Gram-negative bacteria, and two strains of Gram-positive bacteria. The most active molecules have been investigated for inhibition assays of DNA gyrase A, and time-killing kinetics research against *E. coli* was conducted. To determine the biological activity of these molecules, we select the most potent ones and analyze their ADME properties using the SwissADME, Molsoft, PreADME, and Datawarrior websites. Additionally, docking studies will be performed on DNA gyrase A.

Results and discussion

Chemistry

This investigation describes a one-step reaction in which diverse aldehydes react with cyanoacetohydrazide, cyanoacetamide, or cyanothioacetamide. Using this approach produced *N*-amino-5-cyano-6-pyridones in high yields through a multicomponent reaction, as Scheme 1 shows. Firstly, equimolar ethyl cyanoacetate was added to hydrazine hydrate at 0 °C while stirring to produce cyanoacetohydrazide **1**. A 95% yield was obtained by filtering, rinsing with ethanol, and drying the solid cyanoacetic acid hydrazide **1** [23]. The synthesis of derivatives of 1,2-diamino-5-cyano-6-oxo-1,6-dihydropyridine-3-carboxamide **3a-e** was carried out by heating cyanoacetohydrazide **1**, cyanoacetamide **2a**, and different aldehydes in a mixture of ethanol and water, with the addition of a small quantity of piperidine as a catalyst [24]. Subsequently, the compound cyanothioacetamide **2b** was combined with cyanoacetohydrazide **1** and various aldehydes. Following this, the mixture was heated at 80 °C under reflux in a solution of ethanol and water. Additionally, a small quantity of piperidine was added as a catalyst. The aforementioned reaction led to the synthesis of the needed compounds **4a-d** [24]. The molecular structures of the newly produced molecules **3a-e** and **4a-d** were determined based on their infrared (IR), proton nuclear magnetic resonance (^{1}H NMR), carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopic, mass spectrometric data, and elemental analyses. These data were found to be in complete agreement with the hypothesized structures. The **IR** spectra of derivatives **3a-e** showed typical bands corresponding to the NH_2 groups in the region of (3396−3185) cm^{-1}. As an illustrative example, the spectrum of **3a** was examined. The infrared spectrum exhibited bands at 3339, 3315, and 3236 cm^{-1} (indicating the presence of $3NH_2$), 2207 cm^{-1} (indicating CN), and 1680, 1669 cm^{-1} (indicating 2 C=O). The **^{1}H NMR** spectrum revealed a new singlet at δ 2.93 *ppm*, which corresponds to CH_3 protons. Additionally, there were D_2O-exchangeable signals at δ 5.86, 7.34, and 7.69 *ppm*, suggesting the existence of three NH_2 protons. The structure was determined by analyzing the **MS** spectrum, which showed a molecular ion peak [M+, 25.52%] at *m/z* 207. The infrared spectrum of **4c** represented distinct bands at specific wavenumbers, namely 3392, 3352, and 3199 cm^{-1} (corresponding to the existence of $3NH_2$), 2223 cm^{-1} (indicating the presence of CN), and 1673 cm^{-1} (representing C=O). The **^{1}H NMR** spectrum of compound **4c** showed a singlet signal at δ 3.04 *ppm*, which was attributed to two CH_3 groups. Additionally, there were three signals at δ 7.63, 8.49 and 9.67 *ppm* that could be exchanged with D_2O, corresponding to three NH_2 protons. The **^{13}C NMR** spectra of **4c** showed a signal at δ 189.93 *ppm*, which corresponds to the C=S carbon. The **MS** spectrum displayed the structure of the compound, featuring the ion peak of the molecule [M+, 5.01%] at *m/z* 328.



Scheme 1 Synthesis of molecules **3a-e** and **4a-d**; Reagents and conditions: (i) Ethanol/ H_2O, piperidine, stirring at 80 °C, 4 h; (ii) acetaldehyde, (iii) 2,4-dichlorobenzaldehyde, (iv) *N*-dimethylaminobenzaldehyde, (v) 4-(benzyloxy)benzaldehyde, (vi) 4-formylphenyl acetate

Table 1 Inhibition zones (mm) of tested molecules towards fungal and bacterial strains

Compound	*S. faecalis*	*B. pumilus*	*E. cloacae*	*E. coli*	*C. albicans*	*S. cerevisiae*
3a	11	12	21	16	12	11
3b	18	19	14	22	9	10
3c	13	16	17	20	12	13
3d	20	20	10	20	10	16
3e	8	19	12	13	12	9
4a	19	16	19	23	19	18
4b	19	15	18	19	13	11
4c	12	10	14	23	17	14
4d	16	17	15	18	10	17
Pen. G	23	26	-	-	-	-
Cipro.	-	-	26	28	-	-
Keto.	-	-	-	-	24	20

Biological studies

The novel molecules were evaluated for antimicrobial properties utilizing the agar well diffusion method against six different microorganisms. The selected pathogenic microorganisms included *Bacillus pumilus* and *Streptococcus faecalis* as Gram-positive bacteria, *Enterobacter cloacae* and *E. coli* as Gram-negative bacteria, and *Saccharomyces cerevisiae* and *Candida albicans* as fungi. Penicillin G served as the reference antibiotic for Gram-positive bacteria, whereas ciprofloxacin was the reference antibiotic for Gram-negative bacteria. Ketoconazole was utilized as the reference antifungal medicine. The broth dilution method was utilized to ascertain the minimum inhibitory concentration (MIC), and minimum bactericidal/ fungicidal concentration (MBC/MFC).

Antimicrobial testing

The obtained preliminary antimicrobial test results (Table 1& Fig. 3) demonstrated that the investigated compounds demonstrated favorable outcomes against specific bacteria and fungus strains. Compound **4a**, which contained 3-carbothioamide and 4-methyl groups attached to the cyanopyridine nucleus, exhibited significant broad-spectrum antimicrobial activity against

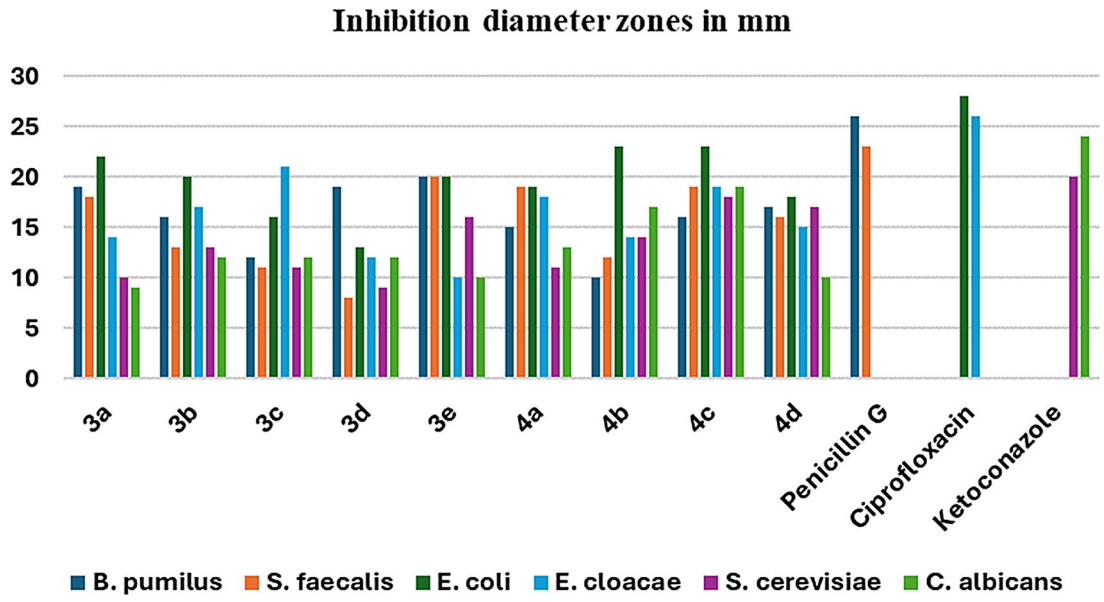


Fig. 3 Inhibition diameter zones in millimeter

the tested bacteria and fungi. Inhibition zones (IZs) for compound **4a** ranged from 16 to 23 mm. In contrast, compound **3a**, which contained 3-carboxamide groups, exhibited diminished activity against all tested bacteria and fungi, with the exception of *E. cloacae* (IZ=21 mm). With inhibition zones (IZs) ranging from 18 to 22 mm, compounds **3b** and **3d** exhibited a notable antibacterial impact against *B. pumilus*, *S. faecalis*, and *E. coli*. In contrast, these compounds demonstrated only mild to moderate activity against *E. cloacae* and two fungal strains, with IZs ranging from 9 to 16 mm. Additionally, **4b** bearing di-chlorophenyl at position 4 of pyridine-carboxamide, was highly active only towards *E. coli*, *S. faecalis*, and *E. cloacae* (IZs=18 &19 mm) and weak against tested fungi. Furthermore, compounds **3c** and **4c** displayed high antimicrobial activity on *E. coli* with inhibition of 20 and 23 mm, respectively and these compounds had mild antimicrobial activity towards other bacterial and fungal strains. Finally, compounds **3e** and **4d** exhibited mild to moderate antimicrobial activity towards tested bacteria and fungi strains.

Evaluation of MIC, MBC, and MFC

The results for the minimum inhibitory concentrations (MIC), the minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) were determined for further investigation. The MBC/MIC and MFC/MIC ratios were determined. The obtained results are illustrated in Tables 2 and 3.

MIC and MBC Regarding Gram-positive Strains: All the analogs exhibited significantly higher inhibitory potency against certain gram-positive bacteria, in comparison to penicillin G as a reference drug. Besides, derivative **4d** bearing phenoxyphenyl at position 4 of pyridine-carbothioamide groups was the most potent antimicrobial against *B. pumilus*, they elicited 8 times more potency than the standard Penicillin G (MIC; 7.81 vs. 62.5 µg / mL). Furthermore, the activity of compounds **3b-d** was found to be four times that of the reference, penicillin G (MIC: 15.6 vs. 62.5 µg/mL). Compounds **3a** and **4c** were 4 times more potent antibacterial against *S. faecalis* than the reference, penicillin G (MIC; 7.81 vs. 31.5 µg / mL). Carboxamide derivatives exhibited a higher degree of antimicrobial efficacy against the tested strains, particularly the tested gram-negative bacteria, compared to carbothioamide derivatives, as seen in Fig. 4.

The study aimed to demonstrate the antimicrobial activities of the examined derivatives, which were found to be bactericidal. The bactericidal effect was observed when the minimum bactericidal concentration was no more than four times the minimum inhibitory concentration value [25, 26]. Our derivatives exhibited a significant MBC/MIC ratio of roughly≤4, showing their strong bactericidal activity. Compounds **3b**, **3e**, and **4a-c** exhibited highly effective bactericidal activity against *B. pumilus*, with MBC values ranging from 31.3 to 125 µg/mL. The MBC/MIC ratios for these compounds were 2, indicating a strong bactericidal effect. In comparison, penicillin had an MBC of 125 µg/mL and an MBC/MIC ratio of 2. Compounds **3a**, **3c**, **3d**, and **4d**, on the other hand, had MBC/MIC ratios of 4. Furthermore, derivatives **3b**, **3c**, and **3e**, along with derivative **4d**, demonstrated enhanced bactericidal efficacy against *S. faecalis*, as evidenced by their respective MBC values of 62.5 and 125 µg/mL. The ratio of MBC to MIC was 2. It is worth mentioning that

Table 2 In vitro MIC, MBC (µg/mL), and MBC/MIC ratio of the tested molecules

Compound	*B. pumilus*			*S. faecalis*			*E. coli*			*E. cloacae*		
	MIC	MBC	MBC/ MIC ratio	MIC	MBC	MBC/ MIC ratio	MIC	MBC	MBC/ MIC ratio	MIC	MBC	MBC/ MIC ratio
3a	31.3	125	4	7.81	31.3	4	15.6	62.5	4	3.91	31.3	8
3b	15.6	31.3	2	31.3	62.5	2	7.81	31.3	4	31.3	125	4
3c	15.6	62.5	4	62.5	125	2	15.6	62.5	4	62.5	125	2
3d	15.6	62.5	4	15.6	62.5	4	3.91	15.6	4	7.81	31.3	4
3e	62.5	125	2	31.3	62.5	2	3.91	15.6	4	15.6	62.5	4
4a	62.5	125	2	15.6	62.5	4	15.6	31.3	2	62.5	125	2
4b	31.3	62.5	2	31.3	125	4	7.81	62.5	4	31.3	125	4
4c	62.5	125	2	7.81	31.3	4	31.3	125	4	62.5	250	4
4d	7.81	31.3	4	31.3	62.5	2	7.81	31.3	4	62.5	125	2
Pen. G	62.5	125	2	31.5	62.5	2	-	-		-	-	
Cipro.	-	-		-	-		<1.95	<1.95		<1.95	<1.95	

the newly synthesized compounds showed strong bactericidal activity towards *E. coli* and *E. cloacae* with MBC/MIC ratios: 2 and 4 except compound **3a** which has an MBC/MIC ratio equal to 8.

Structure-activity relationship study Table 2 present a summary of the MIC values of Carboxamide and carbothioamide derivatives (**3a-e** and **4a-e**) against selected gram-negative and gram-positive bacteria in addition to two types of fungi. Most of our compounds exhibited a promising activity against *E.coil.* Based on the acquired MIC values against *E.coil*, it can be concluded that carboxamido compounds **3d** and **3e** exhibited the highest efficacy of *E. coli* (MIC=3.91 µg/mL). In contrast, compound **4d** demonstrates 7.81 µg/mL against the *E.coli*. Substituting benzyloxy group in compound **3d** with an electron-donating dimethyl amino group in compound **3c** results in a decrease in activity against *E.coli*, as seen by an increase in MIC value from 3.91 µg/ml to 15.6 µg/ml. Carboxamide compound **3c**, which possesses stronger electronegative atoms (dichloro) exhibited MIC value (15.6 µg/mL) two-folds that of its counterpart carbothioamido **4c** (31.3 µg/mL). To summarize the relation between the effect of the substitution at position 4 within carboxamides **3a-e**, the anti-*E-coli* activity was increased in the order of benzyloxy phenyl=phenyl acetate>2,4di-Cl benzene>CH_3=*N*-dimethylaminonenzene. Finally, the effect of 4 substitution on the anti-*E-coli* activity of carbothioamido derivatives **4a-d** increased in the following order: 2,4-di-Cl benzene=benzyloxy phenyl>CH_3>*N*-dimethylaminonenzene.

MIC and MFC The compounds that were evaluated showed generally week efficacy against the fungus strains that were studied (Table 3). Substantial fungicidal effectiveness against *C. albicans* was demonstrated by compound **4c**, which had a MIC of 15.6 µg/mL and MFC to MIC ratio of 4. Concurrently, compounds **3a**, **3c**, **3d**, and **4d** demonstrated noteworthy fungicidal efficacy towards *C. albicans*, as evidenced by their MIC values of 31.3 µg/mL. The MIC to minimal fungicidal concentration (MFC) ratio was 2, while the MFC was 4.

Inhibitory effects against gyrase A enzyme

The DNA gyrase A undergoes negative supercoiling due to the activity of an enzyme. It was proposed that it has a role in transcription and replication in both the initiation and elongation stages in bacterial species [27]. As a result, the inhibition of its activity by a new inhibitor presents a special advantage concerning the emergence of bacterial resistance. The inhibitory behaviors and mechanisms of the most active derivatives, **3d** and **3e**, against DNA gyrase A isolated from *E. coli* were assessed. Table 4 displays the acquired data as IC_{50} values of

Table 3 In vitro MIC, MFC (µg/mL), and MFC/MIC ratio of the tested molecules

Compound	*S. cerevisiae*			*C. albicans*		
	MIC	MFC	MFC/ MIC ratio	MIC	MFC	MFC/ MIC ratio
3a	31.3	125	4	125	500	4
3b	125	250	2	62.5	125	2
3c	31.3	125	4	62.5	125	2
3d	31.3	62.5	2	125	500	4
3e	62.5	125	2	62.5	125	2
4a	62.5	500	8	31.3	62.5	2
4b	125	500	4	31.3	62.5	2
4c	62.5	125	2	15.6	62.5	4
4d	31.3	62.5	2	62.5	125	2
Ketoconazole	1.95	15.6		1.95	31.3	

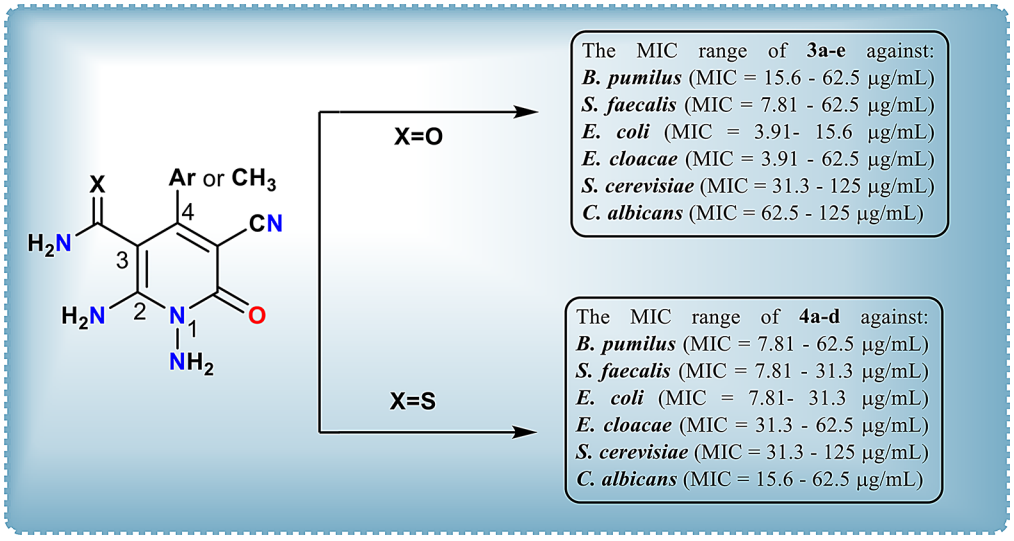


Fig. 4 SAR of the antimicrobial activity of **3a-e** and **4a-d**

Table 4 In Vitro inhibitory activities of the tested molecules **3d** and **3e** against DNA gyrase a enzyme

Cpd No.	IC_{50} ± SD µg/mL	IC_{50} ± SD µM	WI-38 IC_{50} ± SD µM
3d	1.687 ± 0.09	4.48	48.8 ± 2.07
3e	3.771 ± 0.2	11.5	43.2 ± 1.70
Ciprofloxacin	0.455 ± 0.02	1.37	23.9 ± 1.58 [19]

enzyme inhibition in µg/mL. The MICs and IC_{50} exhibited a strong association (Tables 2 and 4), suggesting that these derivatives' inhibition of DNA gyrase resulted in the suppression of bacterial cell growth. The derivatives **3d** and **3e**, which were the most active, demonstrated the ability to inhibit DNA gyrase, with IC_{50} values of 1.68 and 3.77 µg/mL, respectively.

The in vitro cytotoxicity of compounds **3d** and **3e** on the normal WI38 cells was examined as well using the MTT assay to evaluate their safety profiles. Table 4 shows that both substances, **3d** (IC_{50} 48.8 µM) and **3e** (IC_{50} 43.2 µM), were safer than the standard drug, ciprofloxacin (IC_{50} 23.9 µM).

Time-kill kinetic test

The kinetics of derivatives **3d** and **3e**'s killing of bacteria against *E. coli* were examined in order to investigate their antibacterial activity in more detail (Fig. 5). Compound **3d** exhibited bactericidal activity against *E. coli* after a duration of 90 min. Furthermore, during 120 min of incubation, molecules **3e** and gentamycin (1x MIC) demonstrated notable bactericidal activity against *E. coli*.

Molecular docking and in silico calculations

In silico prediction of physicochemical, pharmacokinetic, mutagenicity, tumorigenicity studies, and solubility value

Pharmacokinetic data obtained in silico offer preliminary but important insights into how a drug will ultimately be metabolized by the human body. An oral pharmaceutical candidate is considered to meet Lipinski's criterion if it has a LogP value of 5 or less, a molecular weight (MW) of 500 or less, no more than 5 hydrogen bond donors, and no more than 10 hydrogen bond acceptors [28]. Furthermore, polar surface area (TPSA) rather than the quantity of hydrogen bonding groups could be taken into

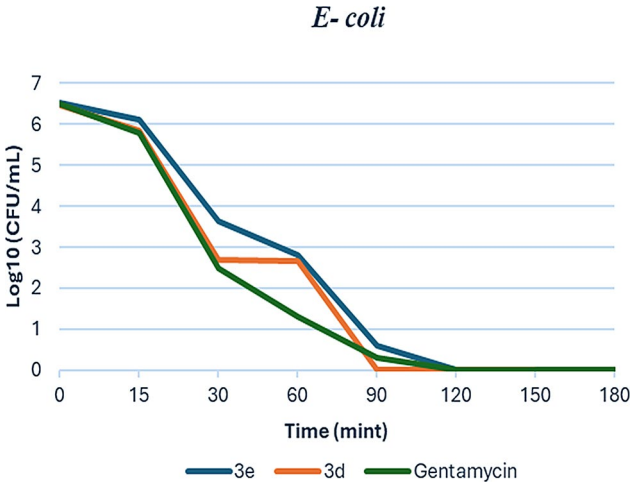


Fig. 5 Time-kill kinetics of molecules **3d**, **3e**, and gentamycin at 1xMIC towards *E. coli*

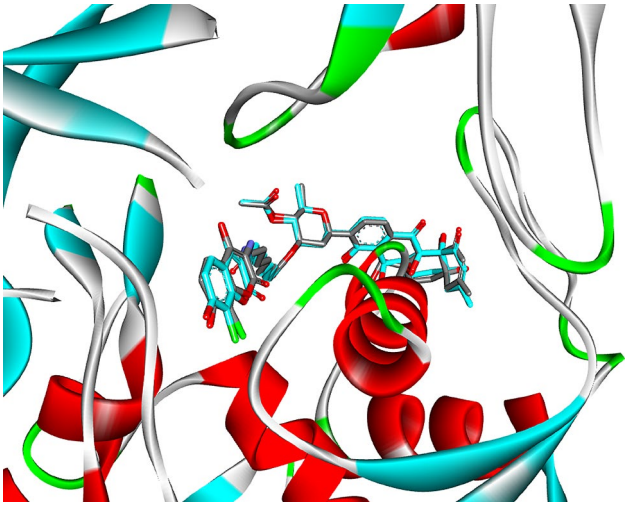


Fig. 6 Co-crystal component (Simocyclinone) superposed in its docked position

consideration. Rats with TPSA of less than 140 Å2 ought to have good oral bioavailability. The pharmacokinetics of the most active drugs, **3d** and **3e**, were assessed using SwissADME software: [29], Molsoft [30], Pre-ADMET [31] and Data Warrior [30]. It was discovered that there is a Lipinski zero violation in the two compounds' physicochemical parameters (Table S1). Furthermore, TPSA values are 150.15 for **3d** and 167.22 $Å^2$ for **3e**. The results indicated that the calculated absorption percentages (ABS) for the synthesized derivatives were 57.19% and 57.19% respectively. This suggests that these derivatives may have the required bioavailability and ability to pass through cell membranes. Molecular **3d** and **3e** contained five and four rotatable bonds, respectively, which signifies their molecular adaptability with respect to their biotarget. One well-known factor that has a big impact on distribution and absorption characteristics is aqueous solubility. With hits of 16323.99 and 20701.67 mg/L (greater than 0.0001 mg/L), respectively, **3d** and **3e** met the solubility requirements. Similarly, human plasma protein binding (PPB), brain-blood barrier partition coefficient (BBB), human intestinal absorption, and Caco2 (colon adenocarcinoma) permeability coefficient were all evaluated in silico using Pre-ADMET software (Table S1).

In the Caco-2 cell model, **3d** and **3e** displayed medium cell permeability, with values ranging from 21.06 to 21.10 nm/s, respectively. The compounds were well-absorbed, as evidenced by the good human intestine absorption values (51.80–87.45%). Moreover, they failed to breach BBB. It was discovered that the two derivatives had modest binding rates (24.78–76.06%) to human plasma proteins. The objective of toxicity risk assessment is to identify chemical structure substructures that may indicate a toxicity risk in any of the three main toxicity classes: mutagenicity, irritancy, and mutagenicity. The mutagenicity, carcinogenicity, and tumorigenicity profiles of the chosen compounds were examined using the DataWarrior program; none of them showed any of these characteristics. We discovered that compound **3e** was not a P-gp protein substrate (Table S1), suggesting that compound **3e** had a very low probability of effluxing out of the cell and having a maximum effect. The Synthetic Accessibility (SA) Score, which is assigned by SwissADME, measures the ease of synthesis of a molecule. The grading scale is standardized and spans from 1 (representing the most challenging level of difficulty) to 10 (representing the least challenging level of difficulty) [29]. The large-scale synthesis of **3d** and **3e** was shown to be feasible, as evidenced by their respective SA scores of 2.83 and 3.11.

Docking into DNA gyrase A's active site

The Protein Data Bank has made available the crystal structure of Gyrase A, which may be accessed using the accession code 4ckl. Docking attempts were conducted utilizing AutoDock Vina, a software application that requires the ligands and receptors to be in the form of pdbqt [32]. Upon redocking the complex enzyme with the co-crystallized ligand, the calculated root mean square deviation (RMSD) between the docked and co-crystallized ligands was 0.44 Å (Fig. 6); thus, the docking method was validated. The findings were visually represented through the utilization of the Discovery Studio 4.5 visualizer [33]. Docking experiments were conducted on molecules **3d** and **3e**, which were identified as the most active in the active site of gyrase A.

DNA gyrase A is critical for preserving the natural topological configuration of DNA in the cell during the process of converting between relaxed and supercoiled forms [34]. The N-terminal domain of GyrA is composed

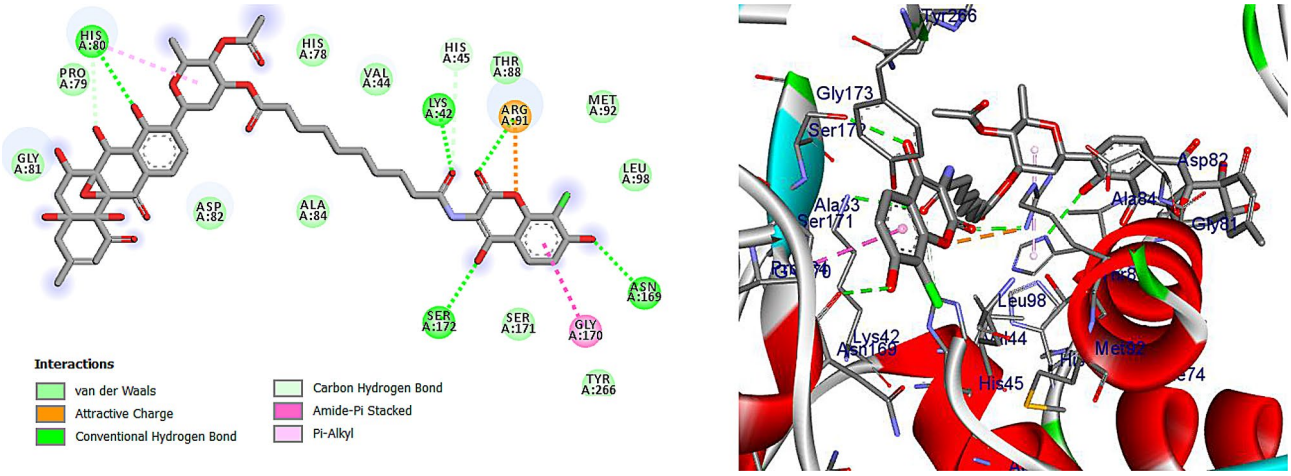


Fig. 7 3D and 2D representations of SD8 in the DNA gyrase A binding region

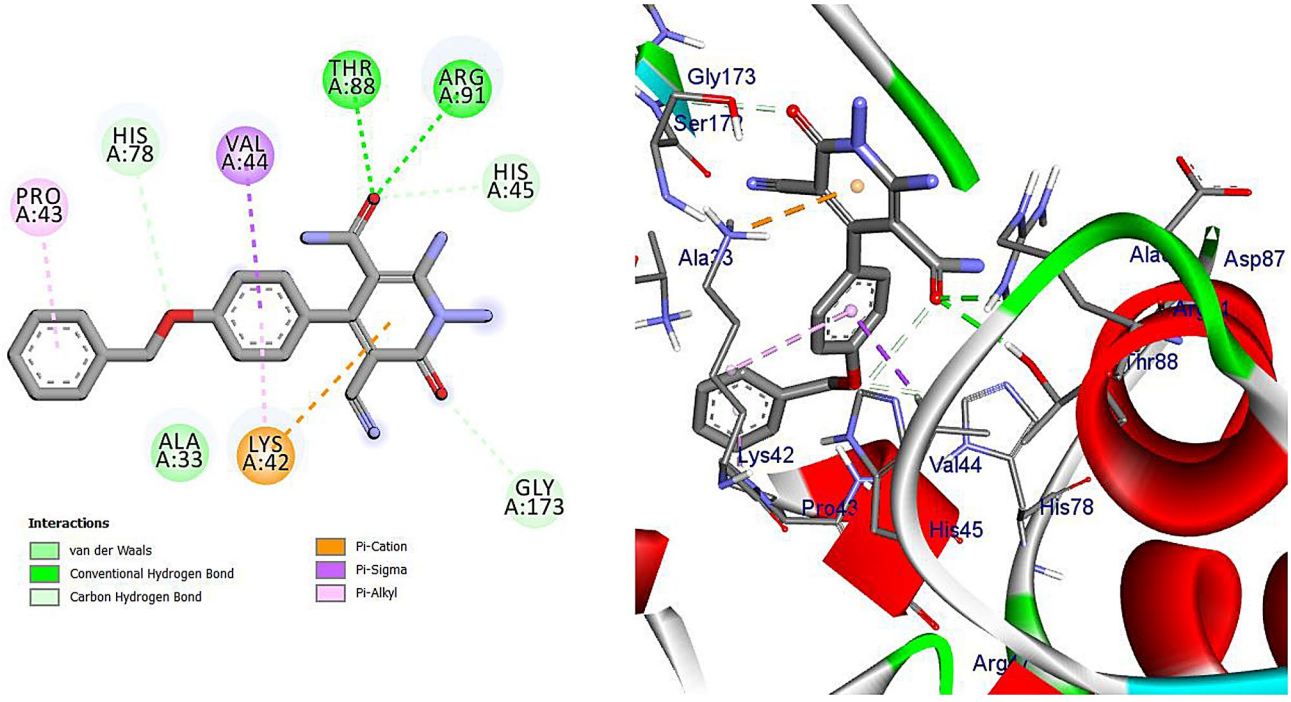


Fig. 8 2D and 3D representations of compound **3d** in the DNA gyrase A binding region

of several critical amino acids, including Arg91, Met120, Val44, Lys42, His80, Gly81, Ser172, Ser97, and His45. These amino acids create a binding region for inhibitors and hinder the binding of DNA [35]. By employing a docking technique, the compounds **3d**, **3e**, and Simocyclinone D8 (SD8) were positioned within the binding domain of the *E. coli* gyrase A enzyme. The docking scores for these molecules were −8.2, -8.3, and −8.2 Kcal/mol, respectively. Simocyclinone established typical hydrogen bonds with Lys42, His 80, Arg91, Ser172, and Asn169. Conversely, it engaged in carbon-hydrogen bonding with His80 and His45, and also formed a hydrophobic interaction with His80. Additionally, it formed an Amide-Pi Stacked connection with Gly170 and Ser171, as depicted in Fig. 7, Table S2. The compounds are attached similarly to the N-terminal domain. The amide group of both compounds established three hydrogen bonds with Thr88, Arg91, and His45. Furthermore, compounds **3d** and **3e** exhibited a hydrophobic contact with Lys42, Val44, and Pro43, contributing to three or more interactions (Figs. 8 and 9, Table S2). In addition, compound **3d** produced two additional hydrogen connections with Gly173 and His78. Compound **3e**, alternatively, exhibited one extra hydrogen bond with Arg47. In addition, ciprofloxacin attached to the active site of gyrase A by establishing four hydrogen bonds with Val44 and Arg47, and

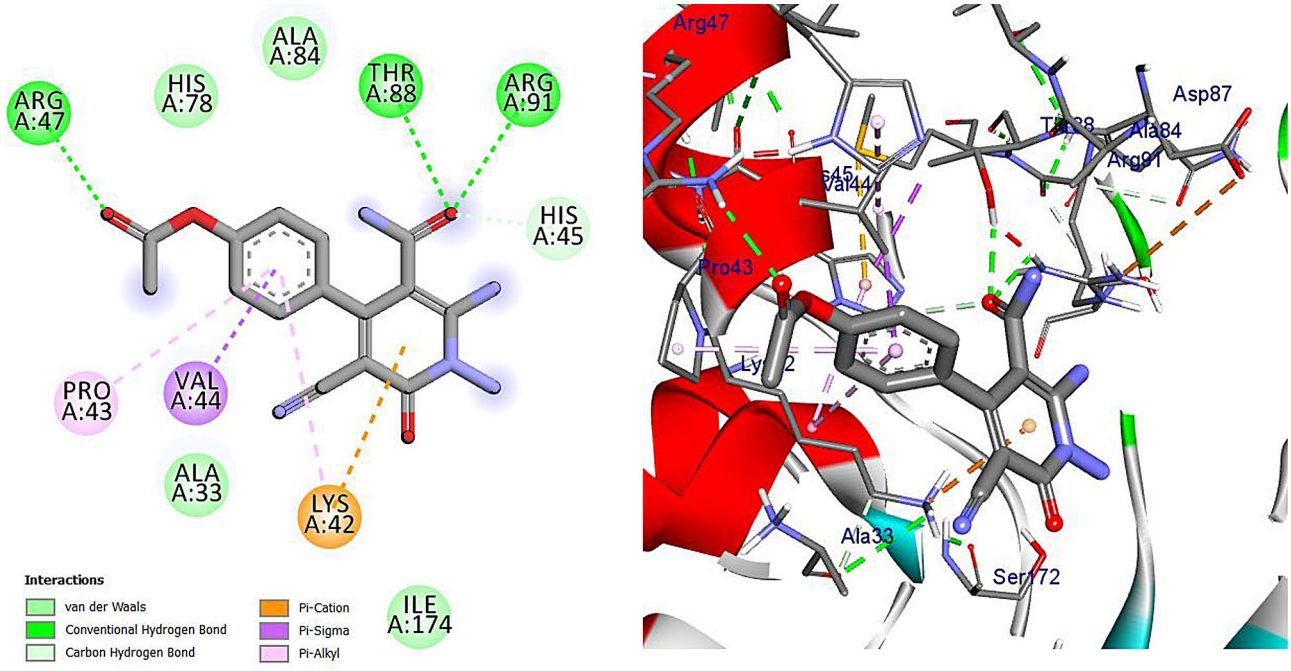


Fig. 9 2D and 3D representations of compound **3e** in the DNA gyrase A binding region

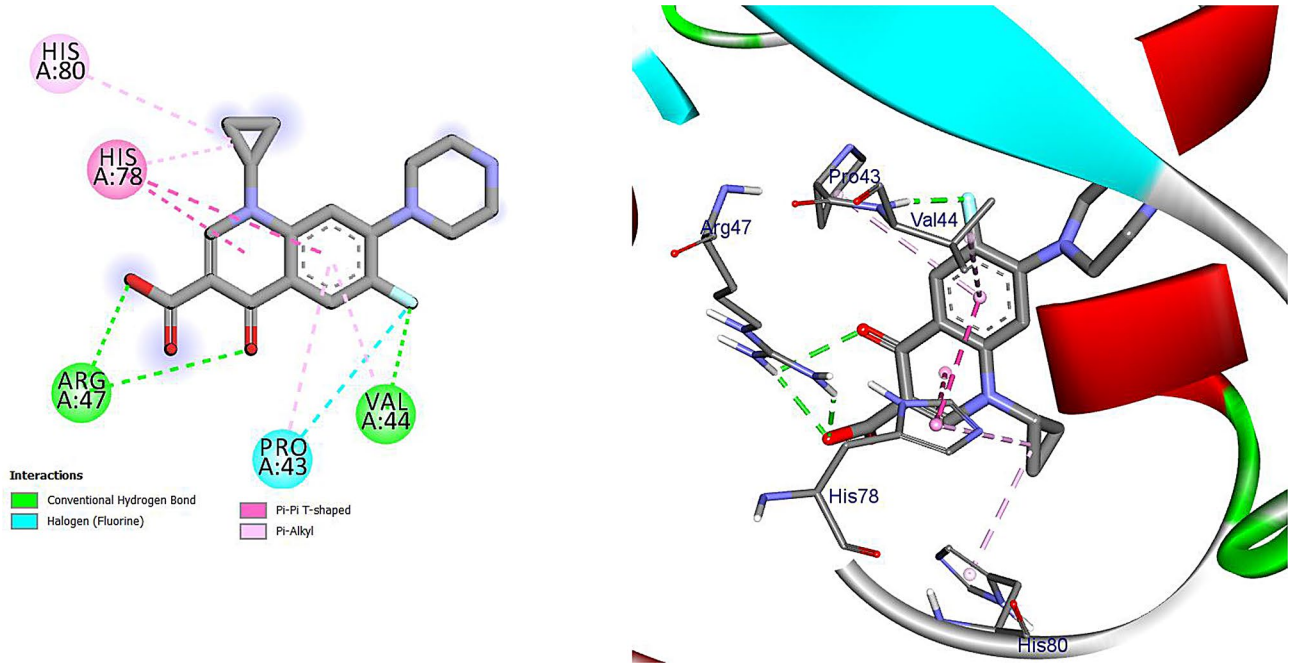


Fig. 10 2D and 3D representations of ciprofloxacin in the DNA gyrase A binding region

it also makes six hydrophobic connections with His78, His80, and Pro43. The fluorine atom formed a halogen connection with Pro43, as shown in Fig. 10 and Table S2. The docking score of ciprofloxacin was −7.7 kcal/mol.

Materials and methods

Chemistry

Instruments and Material (see supplementary file).

The data is available in the supplementary file.

General procedure for the synthesis of 1,2-diamino-5-cyano-6-oxo-4-(substituted phenyl)-1,6-dihydropyridine-3-carboxamide. (3a-e)

At a temperature of 80 °C, an equimolar solution of piperidine (20% mol), cyanoacetohydrazide **2** (1 mmol), cyanoacetamide **4** (1 mmol), and various aldehydes (1 mmol) was stirred within a solution of ethanol and H_2O. After the reaction was completed, as monitored by thin-layer chromatography (TLC) using a 1:1 ratio of ethyl acetate to n-hexane, the solid product was filtered and then washed with a solution of water and ethanol (H_2O/EtOH). This process resulted in the production of pure products **3a-e**.

1,2-diamino-5-cyano-4-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (3a)

Yield 70%; m.p. 140 -142ºC; **IR** (KBr, cm^{-1}): 3339, 3315, 3236 ($3NH_2$), 3053 (CH aromatic),2989 (CH aliphatic), 2207 (C≡N), 1680, 1669(2 C=O), 1583 (C=C); **^{1}H NMR** (DMSO-d_6)δ(ppm): 2.93 (s, 3 H, CH_3), 5.86 (s, 2 H, NH_2;exchangeable with D_2O), 7.34 (s, 2 H, NH_2; exchangeable with D_2O), 7.69 (s, 2 H, NH_2; exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 18.61, 91.18, 116.39, 117.41, 153.08, 157.15, 164.22, 174.59; **MS**, m/z: 207 (M^+, 25.52%); Anal. Calcd. For $C_8H_9N_5O_2$ (207): C, 46.38; H, 4.38; N, 33.80,Found: C, 46.28; H, 4.40; N, 33.81.

1,2-diamino-5-cyano-4-(2,4-dichlorophenyl)-6-oxo-1,6-dihydropyridine-3-carboxamide (3b)

Yield 79%; m.p. 150 -152ºC; **IR** (KBr, cm^{-1}): 3352, 3332, 3212 ($3NH_2$), 3063 (CH aromatic), 2949 (CH aliphatic),2224 (C≡N), 1674, 1659 (2 C=O),1585 (C=C); **^{1}H NMR** (DMSO-d_6) δ (ppm): 5.72 (s, 2 H, NH_2; exchangeable with D_2O), 7.44 (d, H, H_6 Ar-H, *J*=8 Hz), 7.65 (s, H, H_3 Ar-H), 8.28 (d, 2 H, H_5 Ar-H, *J*=8 Hz), 8.47 (s, 2 H, NH_2; exchangeable with D_2O), 9.89 (s, 2 H, NH_2; exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 95.35, 114.45, 126.55, 128.06, 131.26, 131.34, 133.53, 133.69, 134.48, 146.31, 160.00, 165.28, 169.61; **MS**, m/z: 337 (M^+, 35.80%), 339 [$(M+2)^+$, 22.42%], 341 [$(M+4)^+$, 3.33%]; Anal. Calcd. For $C_{13}H_9Cl_2N_5O_2$ (337): C, 46.18; H, 2.68; N, 20.71,Found: C, 46.08; H, 2.71; N, 20.68.

1,2-diamino-5-cyano-4-(4-(dimethylamino)phenyl)-6-oxo-1,6-dihydropyridine-3-carboxamide (3c)

Yield 80%; m.p. 145 -147ºC;**IR** (KBr, cm^{-1}): 3396, 3332, 3252 ($3NH_2$), 3022(CH aromatic),2922 (CH aliphatic),2208 (C≡N), 1678, 1665 (2 C=O), 1570 (C=C); **^{1}H NMR** (DMSO-d_6) δ (ppm): 2.96 (s, 6 H, $2CH_3$), 6.68, (d, 2 H, $H_{3,5}$Ar-H, *J*=8 Hz), 7.43 (d, 2 H, $H_{2,6}$Ar-H, *J*=8 Hz), 7.63 (d, 4 H, $2NH_2$; exchangeable with D_2O), 8.50 (s, 2 H, NH_2; exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 43.74, 92.93, 110.41, 111.66, 111.71, 121.25, 121.55, 130.71, 152.00, 159.84, 161.93, 163.58; **MS**, m/z: 312 (M^+, 42.52%); Anal. Calcd. For $C_{15}H_{16}N_6O_2$ (312): C, 57.68; H, 5.16; N, 26.91,Found: C, 47.58; H, 5.18; N, 26.92.

1,2-diamino-4-(4-(benzyloxy)phenyl)-5-cyano-6-oxo-1,6-dihydropyridine-3-carboxamide (3d)

Yield 63%; m.p. 90 -92ºC;**IR** (KBr, cm^{-1}): 3321, 3332, 3185($3NH_2$),3002(CH aromatic),2933(CH aliphatic),2216(C≡N), 1677, 1669(2 C=O), 1585(C=C); **^{1}H NMR** (DMSO-d_6) δ (ppm): 5.02 (d, 2 H, OCH_2), 6.90 (d, 2 H, $H_{3,5}$ Ar-H, *J*=12 Hz), 7.04 (d, 2 H, $H_{2,6}$ Ar-H, *J*=12 Hz), 7.34–7.43 (m, 5 H, phenyl Hs), 7.82 (d, 4 H, $2NH_2$; exchangeable with D_2O), 8.64 (s, 2 H, NH_2; exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 69.20, 84.78, 113.35, 114.30, 114.89, 126.46, 127.80, 128.45, 129.05, 130.91, 137.09, 150.50, 157.03, 159.02, 169.22, 172.33; **MS**, m/z: 375 (M^+, 13.65%); Anal. Calcd. For $C_{20}H_{17}N_5O_3$ (375): C, 63.99; H, 4.56; N, 18.66,Found: C, 63.97; H, 4.55; N, 18.67; HPLC: rt 8.12 min (purity>95.0%).

4-(1,6-diamino-5-carbamoyl-3-cyano-2-oxo-1,2-dihydropyridin-4-yl)phenyl acetate (3e)

Yield 54%; m.p.280 -280ºC; **IR** (KBr, cm^{-1}): 3334, 3313, 3201($3NH_2$), 3025(CH aromatic), 2987(CH aliphatic), 2208(C≡N), 1679, 1663(2 C=O), 1597(C=C); **^{1}H NMR** (DMSO-d_6) δ (ppm): 2.50 (s, 3 H, CH_3), 6.62, (d, 2 H, $H_{3,5}$ Ar-H, *J*=8 Hz), 7.18 (d, 2 H, $H_{2,6}$ Ar-H, *J*=8 Hz), 7.46 (s, 2 H, NH_2; exchangeable with D_2O), 8.42 (s, 2 H, NH_2; exchangeable with D_2O); 9.09 (s, 2 H, NH_2;exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 23.94, 89.57, 113.63, 114.90, 126.26, 128.11, 129.88, 151.53, 155.92, 157.59, 157.44, 165.77, 172.72; **MS**, m/z: 327 (M^+, 21.29%);Anal.Calcd. For $C_{15}H_{13}N_5O_4$ (327):C, 55.05;H,4.00; N, 21.40, Found: C, 55.06; H, 3.99; N, 21.42; HPLC: rt 8.11 min (purity>95.0%).

General procedure for the synthesis of 1,2-diamino-5-cyano-6-oxo-4-(substituted phenyl)-1,6-dihydropyridine-3-carbothioamide. (4a-d)

The combination containing 1 mmol of cyanoacetohydrazide **2**, 1 mmol of cyanothioacetamide **4**, 1 mmol of aromatic aldehyde, and 20% mol of piperidine in a mixture of ethanol-water was agitated at 80 °C. Following the conclusion of the procedure, which was observed using thin-layer chromatography (TLC) with a mixture of ethyl acetate and n-hexane in a 1:1 ratio, the resulting product was obtained by filtering and subsequently rinsed with a mixture of water and ethanol (H_2O/EtOH) to yield the pure products **4a-d**.

1,2-diamino-5-cyano-4-methyl-6-oxo-1,6-dihydropyridine-3-carbothioamide (4a) Yield 80%;m.p.230–232 oC; IR (KBr, cm−1): 3344, 3320, 3230 (3NH2),

3021(CH aromatic), 2954(CH aliphatic), 2201(C≡N), 1665(C=O), 1587(C=C);; 1H NMR (DMSO-d6) δ(ppm): 2.51 (s, 3 H, CH3), 5.28 (s, 2 H, NH2; exchangeable with D2O), 6.24 (s, 2 H, NH2; exchangeable with D2O); 7.56 (s, 2 H, NH2; exchangeable with D2O); 13C NMR (DMSO-d6) δ (ppm): 18.56, 72.05, 115.55, 117.35, 143.70, 158.30, 161.46, 190.35; MS, m/z: 223 (M+, 11.69%); anal. Calcd. For C8H9N5OS(223): C, 43.04; H, 4.06; N, 31.37, S, 14.36, found: C, 43.06; H, 4.05; N, 31.40; S, 14.39

1,2-diamino-5-cyano-4-(2,4-dichlorophenyl)-6-oxo-1,6-dihydropyridine-3-carbothioamide (4b) Yield 72%;m.p.204–206 °C;**IR** (KBr, cm^{-1}): 3364, 3342, 3208 ($3NH_2$), 3019(CH aromatic), 2939(CH aliphatic), 2191(C≡N), 1653(C=O),1550(C=C); **^{1}H NMR** (DMSO-d_6) δ (ppm): 7.53 (d, H, H_6 Ar-H, J=8 Hz), 7.69 (s, 2 H, NH_2;exchangeable with D_2O), 7.78 (s, H, H_3 Ar-H), 8.11 (d, 2 H, H_5 Ar-H, J=8 Hz), 8.89 (s, 2 H, NH_2;exchangeable with D_2O), 10.27 (s, 2 H, NH_2;exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 75.60, 116.30, 117.65, 125.25, 127.09, 128.13, 129.28, 133.40, 135.64, 145.73, 160.55, 165.22, 190.12; **MS**, m/z: 353 (M^+, 20.23%), 355 ([(M+2)$^+$], 15.14%), 357 ([(M+4)$^+$], 2.04%); Anal. Calcd. For $C_{13}H_9Cl_2N_5OS$ (353): C, 44.08; H, 2.56; N, 19.77;S, 9.05, Found: C, 44.10; H, 2.57;N, 19.15; S, 9.09.

1,2-diamino-5-cyano-4-(4-(dimethylamino)phenyl)-6-oxo-1,6-dihydropyridine-3-carbothioamide (4c) Yield 80%;m.p. 260 -262°C; **IR** (KBr, cm^{-1}): 3392, 3352, 3199 ($3NH_2$), 3082(CH aromatic), 2985(CH aliphatic), 2223(C≡N), 1673(C=O), 1557(C=C);**^{1}H NMR** (DMSO-d_6) δ (ppm): 3.04 (s, 6 H, $2CH_3$), 6.78 (d, 2 H, $H_{3,5}$ Ar-H, J=8 Hz), 7.63 (s, 2 H, NH_2; exchangeable with D_2O), 7.67 (d, 2 H, $H_{2,6}$ Ar-H, J=8 Hz), 8.49 (s, 2 H, NH_2; exchangeable with D_2O), 9.67 (s, 2 H, NH_2; exchangeable with D_2O);**^{13}C NMR** (DMSO-d_6) δ (ppm): 43.81, 73.65, 111.10, 111.71, 115.80, 124.53, 131.59, 145.73, 151.32, 163.43, 169.59, 189.93; **MS**, m/z: 328 (M^+, 5.11%); Anal. Calcd. For $C_{15}H_{16}N_6OS$ (328): C,54.86; H,4.91; N, 25.59; S, 9.76, Found: C, 54.84; H, 2.57; N, 25.57; S, 9.73.

1,2-diamino-4-(4-(benzyloxy)phenyl)-5-cyano-6-oxo-1,6-dihydropyridine-3-carbothioamide (4d) Yield 70%;m.p. 150–152°C; **IR** (KBr, cm^{-1}):3375, 3352, 3256 ($3NH_2$), 3027(CH aromatic), 2999(CH aliphatic), 2209(C≡N), 1672(C=O), 1594(C=C); **^{1}H NMR** (DMSO-d_6) δ (ppm): 5.18 (s, 2 H, OCH_2), 7.20, (d, 2 H, $H_{3,5}$Ar-H, J=8 Hz), 7.34–7.48 (m, 5 H, phenyl Hs, 2 H, NH_2; exchangeable with D_2O), 7.86 (d, 2 H, $H_{2,6}$Ar-H, J=8 Hz), 8.63 (d, 2 H, NH_2;exchangeable with D_2O), 9.87 (s, 2 H, NH_2;exchangeable with D_2O); **^{13}C NMR** (DMSO-d_6) δ (ppm): 69.33, 75.55, 114.19, 115.02, 115.21, 127.42, 128.07, 129.78, 131.80, 136.30, 142.12, 155.20, 160.48, 163.28, 169.90, 191.29; **MS**, m/z: 391 (M^+, 11.59%); Anal.Calcd. For $C_{20}H_{17}N_5O_2S$ (391):C,61.73;H,4.38;N,17.89; S,8.19, Found: C,61.70; H,4.36; N,17.91; S,8.20.

Biological evaluations

Antimicrobial, MIC, MBC, MFC assessment

In order to evaluate the effectiveness of newly produced molecules in preventing the growth of the studied microorganisms, an agar diffusion test was conducted on them. The analytical results were provided in respect to the dimensions of the produced inhibitory zones [36, 37]. MIC, MFC, and MBC assays were performed in accordance with the procedures outlined in the cited articles [19, 38, 39] (See the supplemental file).

DNA gyrase supercoiling assay [40]

(See supplementary file).

Time-kill curve calculation towards E. Coli [41]

(see supplementary file)

In vitro cytotoxicity assay [42]

(see supplementary file)

Conclusion

The primary objective of this investigation was to assess the antimicrobial effectiveness of carboxamide derivatives (**3a-e**) and carbothioamide compounds (**4a-d**). Most of the tested compounds exhibited a notably potent antibacterial action against the chosen Gram-positive bacteria in comparison to the standard reference drug. Moreover, the investigated gram-negative bacteria, particularly *E. coli*, experienced substantial inhibition from compounds **3b**, **3d**, **3e**, **4b**, and **4d**. Furthermore, all evaluated analogs exhibited a satisfactory to moderate level of antifungal effectiveness. Compound **3d** and compound **3e**, which showed the most potential, were evaluated for their ability to impede DNA Gyrase A. Compound **3d** exhibited significant inhibitory activity, with an IC_{50} value of 1.68 µg/mL. Moreover, the study of the kinetics at which compounds **3d** and **3e** eliminate bacteria showed that they exhibit bactericidal activity within 90–120 min of being exposed to *E. coli*. In addition, compounds **3d** and **3e** exhibited favorable pharmacokinetic characteristics. Furthermore, the docking studies revealed a robust affinity for the DNA Gyrase A binding site.

Abbreviations

MIC	Minimum Inhibitory Concentrations
E.coli	Escherichia coli
P. aeruginosa	Pseudomonas aeruginosa
C. albicans	Candida albicans
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
IR	Infrared
B. pumilus	Bacillus pumilus
S. faecalis	Streptococcus faecalis
S. cerevisiae	Saccharomyces cerevisiae

E. cloacae	Enterobacter cloacae
IZ	Inhibition Zone
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
SAR	Structure-Activity Relationship
TPSA	Topological Polar Surface Area
BBB	Brain-Blood Barrier
PPB	Plasma Protein Binding
SA	Synthetic Accessibility
RMSD	Root Mean Square Deviation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13065-024-01342-9.

Supplementary Material 1

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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