



Synthesis and biological evaluation of rhodanine derivatives bearing a quinoline moiety as potent antimicrobial agents

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ABSTRACT

Three series of rhodanine derivatives bearing a quinoline moiety (**6a–h**, **7a–g**, and **8a–e**) have been synthesized, characterized, and evaluated as antibacterial agents. The majority of these compounds showed potent antibacterial activities against several different strains of Gram-positive bacteria, including multidrug-resistant clinical isolates. Of the compounds tested, **6g** and **8c** were identified as the most effective with minimum inhibitory concentration (MIC) values of 1 µg/mL against multidrug-resistant Gram-positive organisms, including methicillin-resistant and quinolone-resistant *Staphylococcus aureus* (MRSA and QRSA, respectively). None of the compounds exhibited any activity against the Gram-negative bacteria *Escherichia coli* 1356 at 64 µg/mL. The cytotoxic activity assay showed that compounds **6g**, **7g** and **8e** exhibited in vitro antibacterial activity at non-cytotoxic concentrations. Thus, these studies suggest that rhodanine derivatives bearing a quinoline moiety are interesting scaffolds for the development of novel Gram-positive antibacterial agents.

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The treatment of bacterial infections capable of causing serious and widespread diseases still remains a significant and worldwide problem because of problems associated with the emergence of new infectious diseases and the increased number of pathogenic microorganisms that are developing resistance to the existing drugs.^{1–5} So development of novel antimicrobial drugs with different mechanisms of action to the currently available antibacterial drugs is still in demand.^{6,7}

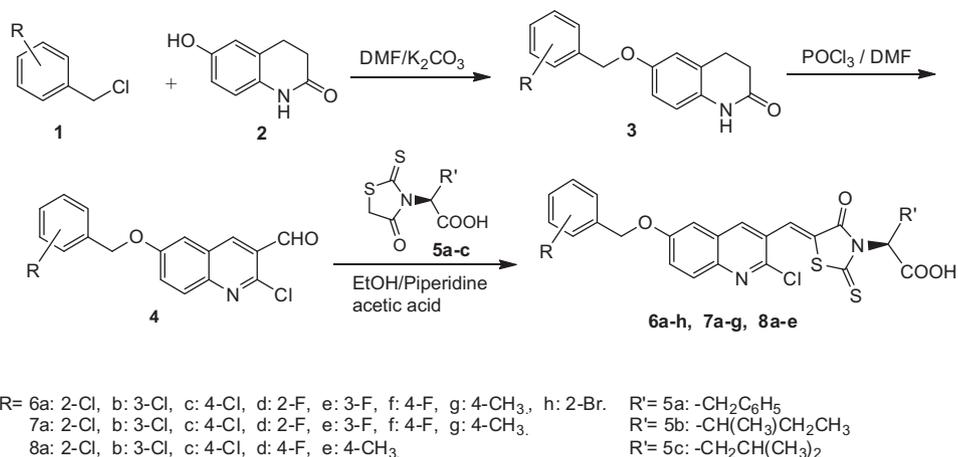
A significant number of compounds bearing a quinoline moiety have been reported in the literature with a variety of different pharmacological activities, including antimicrobial,^{8–10} antituberculosis,¹¹ anticancer,¹² anti-HIV,¹³ antimalarial,¹⁴ and anti-inflammatory activities.¹⁵ Furthermore, rhodanine-based molecules have been reported to be associated with antibiotic activity.¹⁶ In our previous work, we found that several rhodanine derivatives bearing chalcone,¹⁷ 1,3-diarylpyrazole,⁶ (2-oxo-2-phenylethoxy)benzylidene,¹⁸ and (benzyloxy)benzylidene¹⁹ moieties showed moderate to strong activities against several Gram-positive bacterial strains, including multidrug-resistant clinical isolates. Although, to date, there have been no reports in the literature providing a clear understanding of the mechanisms of action associated with these derivatives, the focus of the present work was to introduce a (quinolin-3-yl)methylidene moiety at the 5-position of the rhodanine ring instead of the benzylidene moiety, with the

aim of modulating the hydrophobicity of the resulting molecule and affecting its enzyme binding affinity. Moreover, the effects of different substituents at the 3-position of the rhodanine ring were simultaneously investigated using aromatic or aliphatic groups to observe their effects on the inhibitory activities of the compounds. Thus, as a part of our ongoing studies towards the development of novel antibacterial agents, herein we report the design, synthesis, and antimicrobial evaluation of three novel series of rhodanine derivatives containing a quinoline moiety as efficient antimicrobial agents.

Nineteen new rhodanine derivatives were synthesized according to the synthetic route depicted in Scheme 1. Williamson condensation reactions between 6-hydroxy-3,4-dihydroquinolin-2(1H)-one (**2**) and a variety of different substituted chloromethylbenzene compounds (**1a–1h**) afforded the corresponding 6-(substitutedbenzyloxy)-3,4-dihydroquinolin-2(1H)-ones (**3a–3h**), which were subsequently reacted under Vilsmeier–Haack conditions to give the corresponding 6-(substitutedbenzyloxy)-2-chloroquinoline-3-carbaldehydes (**4a–4h**). The rhodanine intermediates (**5a–c**) were prepared according to a method previously described in the literature.¹⁷ The target compounds **6a–h**, **7a–g**, and **8a–e** were synthesized by the Knoevenagel condensation reactions of **4** with compounds **5a–5c** in ethanol in the presence of glacial acid and piperidine. The structures of the synthesized compounds were confirmed by Fourier transform infra-red (FTIR), ¹H and ¹³C NMR, and mass spectroscopy.²⁰

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Scheme 1. Synthetic route for the construction of target compounds **6a–6h**, **7a–7g**, and **8a–8e**.

The *in vitro* antimicrobial activities of the newly synthesized compounds were evaluated against eight different bacterial strains, including multidrug-resistant clinical isolates, using a 96-well microtiter plate format and a broth microdilution method to obtain their minimum inhibitory concentration (MIC) values. Norfloxacin, oxacillin, gatifloxacin, and moxifloxacin were used as positive controls against the different bacterial strains.

The newly synthesized compounds were also screened for their antibacterial activity against several different Gram-positive organisms, including *Staphylococcus aureus* RN4220, *S. aureus* KCTC 503, and *S. aureus* KCTC 209, as well as the Gram-negative organism, *Escherichia coli* 1356. As shown in Table 1,²¹ the majority of

the compounds showed potent *in vitro* antibacterial activities against the three different types of Gram-positive bacteria, with MIC values in the range of 1–64 µg/mL. Furthermore, compounds **7f**, **8a**, **8c**, and **8e** were particularly active against the Gram-positive strain *S. aureus* RN4220 with an MIC value of 1 µg/mL in both cases, slightly less active than gatifloxacin and moxifloxacin (MIC = 1 µg/mL). Most of the test compounds presented moderate inhibitory activities against *S. aureus* KCTC 503 (MIC = 4–8 µg/mL). Among the desired compounds, **6c**, **6e**, **6f**, **7b–d**, **7f**, **7g**, and **8c** showed potent inhibitory activities against *S. aureus* KCTC 503 (MIC = 4 µg/mL), which was comparable to gatifloxacin (MIC = 4 µg/mL) but less active than moxifloxacin (MIC = 2 µg/

Table 1
Inhibitory activities (MIC, µg/mL) of compounds **6a–6h**, **7a–7g**, and **8a–8e** against bacteria

| Compound | R | R' | Gram-positive strains <i>S. aureus</i> | | | Gram-negative strains <i>E. coli</i> 1356 |
|--------------|-------------------|--|---|-----|-----|---|
| | | | 4220 | 209 | 503 | |
| 6a | 2-Cl | -CH ₂ C ₆ H ₅ | 4 | 64 | 8 | >64 |
| 6b | 3-Cl | -CH ₂ C ₆ H ₅ | 2 | 64 | 8 | >64 |
| 6c | 4-Cl | -CH ₂ C ₆ H ₅ | 4 | 8 | 4 | >64 |
| 6d | 2-F | -CH ₂ C ₆ H ₅ | 4 | 64 | 8 | >64 |
| 6e | 3-F | -CH ₂ C ₆ H ₅ | 4 | 64 | 4 | >64 |
| 6f | 4-F | -CH ₂ C ₆ H ₅ | 4 | 32 | 4 | >64 |
| 6g | 4-CH ₃ | -CH ₂ C ₆ H ₅ | 2 | 8 | 8 | >64 |
| 6h | 2-Br | -CH ₂ C ₆ H ₅ | 4 | 8 | 8 | >64 |
| 7a | 2-Cl | -CH(CH ₃)CH ₂ CH ₃ | 4 | 64 | 8 | >64 |
| 7b | 3-Cl | -CH(CH ₃)CH ₂ CH ₃ | 2 | >64 | 4 | >64 |
| 7c | 4-Cl | -CH(CH ₃)CH ₂ CH ₃ | 2 | 8 | 4 | >64 |
| 7d | 2-F | -CH(CH ₃)CH ₂ CH ₃ | 2 | 32 | 4 | >64 |
| 7e | 3-F | -CH(CH ₃)CH ₂ CH ₃ | 2 | 32 | 8 | >64 |
| 7f | 4-F | -CH(CH ₃)CH ₂ CH ₃ | 1 | 2 | 4 | >64 |
| 7g | 4-CH ₃ | -CH(CH ₃)CH ₂ CH ₃ | 2 | 4 | 4 | >64 |
| 8a | 2-Cl | -CH ₂ CH(CH ₃) ₂ | 1 | 4 | 8 | >64 |
| 8b | 3-Cl | -CH ₂ CH(CH ₃) ₂ | 2 | 4 | 64 | >64 |
| 8c | 4-Cl | -CH ₂ CH(CH ₃) ₂ | 1 | 4 | 4 | >64 |
| 8d | 4-F | -CH ₂ CH(CH ₃) ₂ | 2 | 8 | 8 | >64 |
| 8e | 4-CH ₃ | -CH ₂ CH(CH ₃) ₂ | 1 | 8 | 8 | >64 |
| Norfloxacin | | | 2 | 2 | 2 | 16 |
| Oxacillin | | | 1 | 1 | 1 | >64 |
| Gatifloxacin | | | 0.25 | 2 | 4 | 16 |
| Moxifloxacin | | | 0.25 | 2 | 2 | >64 |

S. aureus RN 4220, *Staphylococcus aureus* RN 4220; *S. aureus* 503, *Staphylococcus aureus* 503; *S. aureus* 209, *Staphylococcus aureus* 209; *E. coli* 1356, *Escherichia coli* CCARM 1356.

mL), norfloxacin (MIC = 2 µg/mL), and oxacillin (MIC = 1 µg/mL), respectively. With the exception of compound **7b** (MIC >64 µg/mL), most of the compounds tested exhibited good levels of inhibitory activity against *S. aureus* KCTC 209 with MIC values in the range of 2–64 µg/mL, but less active than the control drugs (MIC = 1–2 µg/mL). Unfortunately, none of the compounds showed any inhibitory activity against the Gram-negative strain *E. coli* 1356 at 64 µg/mL.

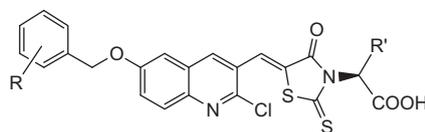
As shown in Table 2,²¹ all of the newly synthesized compounds were also tested for their inhibitory activities against the clinical isolates of several different multidrug-resistant Gram-positive bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA CCARM 3167 and MRSA CCARM 3506) and quinolone-resistant *Staphylococcus aureus* (QRSA CCARM 3505 and QRSA CCARM 3519). With the exception of compound **6f**, compounds **6a–h**, **7a–g**, and **8a–e** showed excellent levels of inhibitory activity against the different multidrug-resistant Gram-positive bacterial strains with MIC values in the range of 1–2 µg/mL. Furthermore, compounds **6g** and **8c** exhibited the strongest levels of inhibitory activity (MIC = 1 µg/mL). This value represented a four to eightfold increase in potency relative to the standard drug norfloxacin (MIC = 4–8 µg/mL) and a four to eightfold increase relative to gatifloxacin and moxifloxacin against quinolone-resistant *Staphylococcus aureus* (MIC = 4–8 µg/mL). These results suggested that the quinoline moiety was an important structural unit and critical to the levels of antibacterial activity observed in the rhodanine-based derivatives investigated during the course of the present study. In addition, some clear structure–activity relationship patterns were found between the antibacterial activity and the physicochemical properties of the N-substituted groups on the rhodanine ring from

Table 1.²⁰ A comparison of the carboxylic acid derivatives at the N-position of the rhodanine ring indicated that the different carboxylic acids contributed to the antimicrobial activity in the order of CH₂CH(CH₃)₂ > CH(CH₃)CH₂CH₃ > CH₂C₆H₅. Furthermore, the position of the substituent on the phenyl ring influenced the anti-bacterial activity with an activity order of 4-F > 3-F, 2-F for fluoro-substituted compounds, and 4-Cl > 3-Cl, 2-Cl for chloro-substituted compounds.

The cytotoxic properties of compounds **6g**, **7g** and **8e** were also investigated using MTT colorimetric assay to determine if their observed antibacterial activity was caused by selective toxicity towards the bacterial cells and the results are shown in Table 3.²² Compounds **6g**, **7g**, and **8e** did not affect cell viability on the Human cervical (HeLa) cells at their MICs but showed cytotoxicity at much higher concentrations. The inconsistency of compounds **6g**, **7g** and **8e** between their antibacterial activity and cytotoxicity suggests that there may be an antibacterial mechanism different from cytotoxicity.

In conclusion, based on our previous work, we synthesized three novel series of rhodanine derivatives containing a quinoline moiety and evaluated their antibacterial activities against Gram-positive and Gram-negative bacteria. The results indicated that the majority of the target compounds exhibited potent antibacterial activity against Gram-positive bacteria, particularly against the multidrug-resistant strains of the clinical isolates. Compounds **6g** and **8c** exhibited a four to eightfold increase in potency relative to the standard drug norfloxacin against all of the selected multidrug-resistant clinical isolates and also a four to eightfold increase relative to gatifloxacin and moxifloxacin against quinolone-resistant *Staphylococcus aureus*. These results suggested that the rhoda-

Table 2
Inhibitory activities (MIC, µg/mL) of compounds **6a–6h**, **7a–7g**, and **8a–8e** against clinical isolates of multidrug-resistant Gram-positive strains



| Compound | R | R' | Multidrug-resistant Gram-positive strains | | | |
|--------------|-------------------|--|---|------|------|------|
| | | | MRSA | | QRSA | |
| | | | 3167 | 3506 | 3167 | 3506 |
| 6a | 2-Cl | –CH ₂ C ₆ H ₅ | 2 | 2 | 2 | 2 |
| 6b | 3-Cl | –CH ₂ C ₆ H ₅ | 2 | 2 | 2 | 2 |
| 6c | 4-Cl | –CH ₂ C ₆ H ₅ | 2 | 2 | 2 | 2 |
| 6d | 2-F | –CH ₂ C ₆ H ₅ | 1 | 2 | 2 | 2 |
| 6e | 3-F | –CH ₂ C ₆ H ₅ | 2 | 1 | 1 | 2 |
| 6f | 4-F | –CH ₂ C ₆ H ₅ | 2 | 2 | 2 | 4 |
| 6g | 4-CH ₃ | –CH ₂ C ₆ H ₅ | 1 | 1 | 1 | 1 |
| 6h | 2-Br | –CH ₂ C ₆ H ₅ | 2 | 2 | 2 | 2 |
| 7a | 2-Cl | –CH(CH ₃)CH ₂ CH ₃ | 2 | 1 | 2 | 2 |
| 7b | 3-Cl | –CH(CH ₃)CH ₂ CH ₃ | 2 | 1 | 1 | 2 |
| 7c | 4-Cl | –CH(CH ₃)CH ₂ CH ₃ | 1 | 1 | 2 | 2 |
| 7d | 2-F | –CH(CH ₃)CH ₂ CH ₃ | 2 | 1 | 2 | 2 |
| 7e | 3-F | –CH(CH ₃)CH ₂ CH ₃ | 2 | 1 | 2 | 2 |
| 7f | 4-F | –CH(CH ₃)CH ₂ CH ₃ | 2 | 1 | 2 | 1 |
| 7g | 4-CH ₃ | –CH(CH ₃)CH ₂ CH ₃ | 2 | 2 | 2 | 2 |
| 8a | 2-Cl | –CH ₂ CH(CH ₃) ₂ | 1 | 1 | 1 | 2 |
| 8b | 3-Cl | –CH ₂ CH(CH ₃) ₂ | 1 | 2 | 2 | 2 |
| 8c | 4-Cl | –CH ₂ CH(CH ₃) ₂ | 1 | 1 | 1 | 1 |
| 8d | 4-F | –CH ₂ CH(CH ₃) ₂ | 2 | 2 | 2 | 1 |
| 8e | 4-CH ₃ | –CH ₂ CH(CH ₃) ₂ | 1 | 2 | 1 | 1 |
| Norfloxacin | | | 8 | 4 | >64 | >64 |
| Oxacillin | | | >64 | >64 | 1 | 1 |
| Gatifloxacin | | | 2 | 1 | 8 | 4 |
| Moxifloxacin | | | 1 | 1 | 4 | 4 |

MRSA 3167, methicillin-resistant *S. aureus* CCARM 3167; MRSA 3506, methicillin-resistant *S. aureus* CCARM 3506; QRSA 3505, quinolone-resistant *S. aureus* CCARM 3505; QRSA 3519, quinolone-resistant *S. aureus* CCARM 3519.

Table 3
Cytotoxic activity of compounds **6g**, **7g** and **8e** against HeLa cell

| Compound | IC ₅₀ (μg/mL) |
|-----------|--------------------------|
| 6g | 13.29 |
| 7g | 9.09 |
| 8e | 8.84 |

nine derivatives bearing a quinoline moiety, which play a critical role in increasing the antibacterial properties of the compounds, represented promising lead compounds for the development of novel antibacterial agents. Compounds **6g**, **7g** and **8e** did not exhibit any significant influence on cell viability in the HeLa cells at their MICs. Further investigations of these compounds are currently underway in our laboratories, including the study of their possible mechanism of action.

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References and notes

- Varshney, V.; Mishra, N. N.; Shukla, P. K.; Sahu, D. P. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3573.
- Muroi, H.; Nihei, K.; Tsujimoto, K.; Kubo, I. *Bioorg. Med. Chem.* **2004**, *12*, 583.
- Woodford, N. *Expert Opin. Investig. Drugs* **2003**, *12*, 117.
- Patel, R. V.; Patel, P. K.; Kumari, P.; Rajani, D. P.; Chikhalia, K. H. *Eur. J. Med. Chem.* **2012**, *53*, 41.
- Jiang, X. J.; Yu, P.; Jiang, J.; Zhang, Z. J.; Wang, Z. L.; Yang, Z. Q.; Tian, Z. M.; Wright, S. C.; Larrick, J. W.; Wang, Y. Q. *Eur. J. Med. Chem.* **2009**, *44*, 2936.
- Xu, L. L.; Zheng, C. J.; Sun, L. P.; Miao, J.; Piao, H. R. *Eur. J. Med. Chem.* **2012**, *48*, 174.
- Khan, M. W.; Alam, M. J.; Rashid, M. A.; Chowdhury, R. *Bioorg. Med. Chem.* **2005**, *13*, 4796.
- Thomas, K. D.; Adhikari, A. V.; Telkar, S.; Chowdhury, I. H.; Mahmood, R.; Pal, N. K.; Row, G.; Sumesh, E. *Eur. J. Med. Chem.* **2011**, *46*, 5283.
- Eswaran, S.; Adhikari, A. V.; Shetty, N. S. *Eur. J. Med. Chem.* **2009**, *44*, 4637.
- Kategaonkar, A. H.; Shinde, P. V.; Kategaonkar, A. H.; Pasale, S. K.; Shingate, B. B.; Shangare, M. S. *Eur. J. Med. Chem.* **2010**, *45*, 3142.
- Lilienkampf, A.; Mao, J.; Wan, B.; Wang, Y.; Franzblau, S. G.; Kozikowski, A. P. *J. Med. Chem.* **2009**, *52*, 2109.
- Denny, W. A.; Wilson, W. R.; Ware, D. C.; Atwell, G. J.; Milbank, J. B.; Stevenson, R. J. U.S. Patent **2006**, 7064117.
- Strekowski, L.; Mokrosz, J. L.; Honkan, V. A.; Czarny, A.; Cegla, M. T.; Patterson, S. E.; Wydra, R. L.; Schinazi, R. F. *J. Med. Chem.* **1991**, *34*, 1739.
- Nasveld, P.; Kitchener, S.; Kitchener, S. *Trans. R. Soc. Trop. Med. Hyg.* **2005**, *99*, 2.
- Leatham, P. A.; Bird, H. A.; Wright, V.; Seymour, D.; Gordon, A. *Eur. J. Rheumatol. Inflamm.* **1983**, *6*, 209.
- Hardej, D.; Ashby, C. R., Jr.; Khadtare, N. S.; Kulkatni, S. S.; Singh, S.; Talele, T. T. *Eur. J. Med. Chem.* **2010**, *45*, 5827.
- Chen, Z. H.; Zheng, C. J.; Sun, L. P.; Piao, H. R. *Eur. J. Med. Chem.* **2010**, *45*, 5739.
- Song, M. X.; Zheng, C. J.; Deng, X. Q.; Wang, Q.; Hou, S. P.; Liu, T. T.; Xing, X. L.; Piao, H. R. *Eur. J. Med. Chem.* **2012**, *54*, 403.
- Jin, X.; Zheng, C. J.; Song, M. X.; Wu, Y.; Sun, L. P.; Li, Y. J.; Yu, L. J.; Piao, H. R. *Eur. J. Med. Chem.* **2012**, *56*, 203.
- Preparation of 8e**: A mixture of **4e** (2 mmol) and **5c** (3 mmol), 10 drops glacial acetic acid and 10 drops piperidine in ethanol (5 mL) was refluxed for 4 h. After cooling, the solvent was evaporated in vacuo, followed by the purification of the resulting residue by silica gel column chromatography (dichloromethane/methanol, 100:1) to get a yellow solid. Yield 35%; mp 96–97 °C. IR (KBr) cm⁻¹: 3436 (OH), 1736 (C=O). ¹H NMR (DMSO-*d*₆, 300 MHz, ppm): δ 0.92 (q, 6H, J = 7 Hz, CH₃), 1.55 (m, 1H, CH), 2.05–2.22 (m, 2H, CH₂), 2.32 (s, 3H, PhCH₃), 5.22 (s, 2H, OCH₂), 5.62 (s, 1H, NCH), 7.95 (s, 1H, Ph = CH), 7.22–8.51 (m, 8H, Ar-H), 13.40 (s, 1H, COOH). MS *m/z* 541 (M+1). ¹³C NMR (DMSO-*d*₆, 75 MHz, ppm): δ 193.20, 169.22, 166.34, 157.41, 147.00, 142.61, 137.48, 137.40, 133.20, 129.03, 128.86, 128.06, 127.84, 127.17, 125.65, 125.35, 125.30, 108.07, 69.81, 56.06, 36.44, 24.81, 22.82, 21.92, 20.78.
- Anti-bacterial activity assay**: The micro-organisms used in the present study were *S. aureus* (*S. aureus* RN 4220, *S. aureus* KCTC 209, *S. aureus* KCTC 503), and *Escherichia coli* (*E. coli* 1356). The strains of multidrug-resistant clinical isolates were methicillin-resistant *Staphylococcus aureus* (MRSA CCARM 3167 and MRSA CCARM 3506) and quinolone-resistant *Staphylococcus aureus* (QRSA CCARM 3505 and QRSA CCARM 3519). Clinical isolates were collected from various patients hospitalized in several clinics. Test bacteria were grown to mid-log phase in Mueller–Hinton broth (MHB) and diluted 1000-fold in the same medium. The bacteria of 10⁵ CFU/mL were inoculated into MHB and dispensed at 0.2 mL/well in a 96-well microtiter plate. As positive controls, oxacillin, norfloxacin, gatifloxacin, and moxifloxacin were used. Test compounds were prepared in DMSO, the final concentration of which did not exceed 0.05%. A twofold serial dilution technique was used to obtain final concentrations of 64–0.5 μg/mL. The MIC was defined as the concentration of a test compound that completely inhibited bacteria growth during 24 h incubation at 37 °C. Bacteria growth was determined by measuring the absorption at 650 nm using a microtiter enzyme-linked immunosorbent assay (ELISA) reader. All experiments were carried out three times.
- Cytotoxicity activity assay**: Human cervical (Hela) cell monolayers were used as an in vitro model of cervicovaginal epithelium for testing the cytotoxicity of the new compounds. Hela cells were grown in Dulbecco modified Eagle medium supplemented with fetal bovine serum (10%), and antibiotics (penicillin–streptomycin mixture [100 U/mL]). Cells at 80–90% confluence were split by trypsin (0.25% in PBS; pH 7.4), and the medium was changed at 24 h intervals. The cells were cultured at 37 °C in a 5% CO₂ incubator. The cells were grown to 3 passages and approximately 1 × 10⁴ cells were seeded into each well of a 96-well plate and allowed to incubate overnight to allow cells to attach to the substrate. After 24 h, the medium was replaced with DMEM supplemented with 10% FBS containing various concentrations of test compounds and incubated for 48 h. Then 10 μL of MTT solution (5 mg/ml in PBS) was added to each well. After incubation for 4 h, the medium was removed and the resulting formazan crystals were dissolved with 100 μL DMSO. After shaking 10 min, the optical density was measured at 570 nm using a microtiter ELISA reader. The assay was conducted four times. The IC₅₀ values were defined as the concentrations inhibiting 50% of cell growth.