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Original Article

Synthesis and Characterization of Chemical Compounds Derived From Benzohydrazide and Evaluation of Their Antibacterial Activities

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Abstract

Background: The antimicrobial resistance of pathogenic bacteria has emerged as a major health problem in recent years. Extensive research has been conducted to find new antimicrobial agents.

Objectives: The aim of this study was to examine the antibacterial activities of benzohydrazide derivatives.

Methods: Manganese hydrogen sulfate choline chloride was applied in a simple method for synthesizing benzohydrazide derivatives. Antibacterial activities of the derivatives were assessed against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Bacillus subtilis*, *diphtheroids*, *Salmonella enterica*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The structure of the synthesized compounds was determined employing $^{1}H/^{13}C$ NMR and Fourier-transform infrared (FT-IR) spectroscopy. The reactions were carried out in choline chloride dissolved in water at room temperature.

Results: The results of this study showed that benzohydrazide derivatives had very desired antibacterial activities against the assessed bacteria.

Conclusions: Further investigations are required to assess the safety and efficacy of benzohydrazide derivatives as antibacterial agents *in vivo* and *in vitro*.

Keywords: Antibacterial activity, Benzohydrazide derivatives, Pathogenic bacteria

Background

Bacterial resistance has become a serious problem because of the widespread use of antibiotics either prophylactically or remedially without proper medical indications. The incorrect selection of alternative antimicrobials and numerous switching between them further contribute to antimicrobial resistance in bacteria (1).

The derivatives of benzohydrazide, a moiety present in the structure of various molecules, have been known to promote important biological activities. For example, studies have investigated pyrrolyl benzohydrazide for antitubercular (2), 2-hydroxy-*N*′-(3-hydroxybenzylidene) benzohydrazide (HHB) for anticancer (3), *N*′-(substituted)-4-(butan-2-ylideneamino) benzohydrazides for *in vitro* antimicrobial and anticancer (4), copper (II), manganese (II), and nickel (II) complexes of (Z)-2-hydroxy-N′-(2-oxoindolin-3-ylidene) benzohydrazide for antitumor (5), [(3,4-disubstituted)-1,3-thiazol-2ylidene]-4-hydroxybenzohydrazide (6) and quinoxalinhydrazides (7) also for antitumor, and finally 2-bromo-5-methoxy-*N*′-[4-(aryl)-1,3-thiazol-2-yl] benzohydrazide derivatives (8) for analgesic, antifungal, and antibacterial activities. In a recent study, the biological effects of three benzohydrazide derivatives were screened, from which two compounds exhibited promising analgesic activities, and one showed *in vitro* antiproliferative activity (8). Furthermore, isonicotinohydrazide and benzohydrazide analogues were evaluated for their *in vitro* antimicrobial activities against *Mycobacterium tuberculosis* $H_{37}Rv$ (MTB) (9). In other studies, benzoheterocyclic analogues of N′-benzoyl-N-(tert-butyl) benzohydrazide showed insecticidal activity against *Spodoptera litura* F. (10), and N′,N′-dibenzyl benzohydrazides did *in vitro* antifungal activity against *Botrytis cinerea* phytopathogenic fungus (11).

The quinoline scaffold is prevalent in the structure of a variety of pharmacologically active synthetic and natural compounds. Quinolines are historically known as the most important antimalarial drugs ever used. Chloroquine which is the most famous drug of this group has dedicated to us great hopes for eradicating malaria. Other known drugs of this family include quinidine, quinine, ciprofloxacin, grepafloxacin, antrafenine, saquinavir, gemifloxacin, topotecan, balofloxacin, and levofloxacin. As a core essential molecule, quinoline in the form of chloroquinolone ring is often used for designing many synthetic compounds with diverse biological activities (12). Some studies, for example, have been conducted to assess 2-chloro-3-((4-phenyl-1*H*-1,2,3-triazol-1-yl) methyl) quinoline derivatives for *in vitro* antifungal and antibacterial effects (13), (2-chloro-quinoline-3-yl)-

methanol for antioxidant and cytotoxic properties (14), 2-chloro-3-(l, 3-dioxolan-2-yl) quinolines for DPPH free radical scavenging activities (15), 2-chloro quinoline-3-carbaldehyde derived Schiff bases for antimicrobial activities (16), 7-chloro-4-quinolinylhydrazone derivatives for antitumoral properties (17), N-(2-(arylmethylimino) ethyl)-7-chloroquinolin-4-amine derivatives for fighting with Zika virus function (18), 1-(7-Chloroquinolin-4-yl)-2-[(1H-pyrrol-2-yl) methylene] hydrazine as an anticancer agent (19), 4-[(p-Aminobenzenesulfonamide-N^1-(2′-pyridinil)]-7-chloroquinoline derivatives as promising antimalarial compounds (20), and finally natural and synthetic chloroquinolone derivatives for *in vivo* and *in vitro* antioxidant features (21). Some review articles have also inspected the chemical reactions, synthetic methods, and biological properties of 2-chloroquinoline-3-carbaldehyde in the articles published between the years 1979-2017 (22-24). This study aimed to examine the antimicrobial effects of synthetic derivatives of benzohydrazide against nine pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Bacillus subtilis*, diphtheroids, *Salmonella enterica*, *Serratia marcescens*, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*.

Methods

The materials were purchased from Merck and Sigma–Aldrich, Germany, and all the chemicals were used without further purification. The composition of the products was characterized by FT-IR, and ^{1}H-NMR, and their physical properties were compared with those reported in the literature. FT-IR spectra were run in a Bruker, Equinox 55 spectrometer. The Bruker (DRX-500 Avanes) NMR device was used to record ^{1}H NMR spectra. Thin-layer chromatography was performed to check the purity of compounds, and the spots were visualized under UV.

Preparation of Green Catalyst Liquid

To prepare manganese hydrogen sulfate choline chloride (MHSCC), $MnCl_2.2H_2O$ (0.1 mol) was poured in a 250-mL round-bottomed flask fitted with a calcium chloride drying tube, and concentrated sulfuric acid (98%, 10.8 mL, 0.2 mol) was added dropwise over 30 minutes at room temperature. After the addition of H_2SO_4, the mixture was shaken for 30 minutes to obtain a pale pink solid. To eliminate H_2SO_4, the solid was washed with absolute ethanol (25). Afterwards, in a 50-mL round-bottomed flask, 5.00 g (0.036 moles) choline chloride was dissolved in 10 mL water, and then the mixture was stirred. Finally, manganese hydrogen sulfate (0.50 g, 2.0 mmol) was added to obtain a transparent liquid.

Preparation of N'-((2-Chloroquinolin-3-yl) Methylene) Benzohydrazides (5a-5h)

Benzohydrazide derivatives (2 mmol) and 2-chloroquinoline-3-carbaldehyde (0.383 g, 2 mmol) were mixed and ground in a pestle. The mixed powder and MHSCC were transferred to a round-bottomed flask and heated up to 60°C. The progress of the reaction was monitored by thin-layer chromatography. After the completion of the reaction, 25 mL ethyl acetate was added to the mixture to precipitate and filter the product. The crude product was then recrystallized from ethanol to form pure benzohydrazide derivatives with the yields of 83% to 95%. All the products were identified by comparing their physical and spectral data with those of authentic samples.

Entry 1: *N'-((2-Chloroquinolin-3-yl) methylene)-2-methoxybenzohydrazide*: Color Light orange, Yield of 93%, mp 144–145 °C.

FT-IR (KBr) νmax/cm^{-1}: 3432, 3266, 2956, 1676, 1649, 1607, 1578. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.10 (1H, s), 9.00 (s, 1H), 8.90 (s, 1H), 8.25 (d, 1H, *J* 8.1 Hz), 8.00 (m, 3H, ArH), 7.90 (t,1H, *J* 8.1Hz), 7.70 (t, 2H, *J* 7.8Hz), 7.10 (d,1H, *J* 9.0 Hz), 3.06 (s, 3H).

Entry 2: *N'-((2-Chloroquinolin-3-yl) methylene)-4-nitrobenzohydrazide*: Color Yellow, Yield of 89%, mp 257–258 °C.

FT-IR (KBr) νmax/cm^{-1}: 3437, 3181, 2853, 1670, 1617, 1597, 1523; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.50 (s,1H), 9.05 (s,1H), 8.95 (s,1H), 8.40 (d, 2H, *J* 8.7 Hz), 8.30 (m, 3H), 8.00 (d,1H, *J* 8.4 Hz), 7.90 (t,1H, *J* 6.9 Hz), 7.70 (t,1H, *J* 7.5 Hz).

Entry 3: *N'-((2-Chloroquinolin-3-yl) methylene)-2-hydroxybenzohydrazide*: Color Off white, Yield of 90%, mp 159–161°C.

FT-IR (KBr) νmax/cm^{-1}: 3196, 3051, 1669, 1590, 1556. ^{1}H NMR (DMSO-d_6, 400 MHz) δ:12.20 (s, 1H, NH), 11.75 (s,1H, OH), 9.00 (s,1H), 8.95 (1H, s), 8.45 (d,1H, *J* 7.5 Hz), 8.00 (d,1H, *J* 8.4 Hz), 7.90 (t, 2H, *J* 7.2 Hz), 7.70 (t,1H, *J* 7.5 Hz), 7.50 (t, 1H, *J* 7.2 Hz), 7.00 (t, 2H, *J* 7.8 Hz).

Entry 4: *N'-((2-Chloroquinolin-3-yl) methylene)-2,4-dinitrobenzohydrazide*: Color Yellow, Yield of 85%, mp 142–143 °C.

FT-IR (KBr) νmax/cm^{-1}: FT-IR (KBr) νmax/cm^{-1}: 3414, 3250, 2916, 1665, 1630, 1619, 1582, 1563. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.40 (1H, s, NH), 8.69 (1H, s), 8.68 (s,1H), 8.26 (d,1H, *J* 8 Hz), 8.00-7.86 (m, 2H), 7.72(t,1H, *J* 8 Hz), 7.57-7.56 (m,1H), 6.19-6.10 (m, 2H).

Entry 5: *N'-((2-Chloroquinolin-3-yl) methylene)-2-(3,5-dichlorophenoxy)acetohydrazide*: Color White, Yield of 88%, mp 199–200 °C.

FT-IR (KBr) νmax/cm^{-1}: 3192, 3113, 2987, 1685, 1635, 1616, 1583. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.10 (s, 1H, NH), 9.03 (s, 1H), 8.70 (s,1H), 8.22 (d,1H, *J* 12 Hz), 7.98 (d,1H, *J* 8 Hz), 7.88 (t,1H, *J* 8 Hz), 7.70-7.34 (m, 3H), 7.20 (m,1H), 5.20 (s, 2H).

Entry 6: *4-Chloro-N'-((2-chloroquinolin-3-yl) methylene) benzohydrazide*: Color Off white, Yield of 85%, mp 268–269 °C.

FT-IR (KBr) νmax/cm^{-1}: 3174, 3053, 2902, 1651, 1618, 1593, 1552. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.40 (s,

1H, NH), 8.95 (s,1H), 8.94 (s,1H), 8.25 (d,1H, *J* 8.1 Hz), 8.00 (m, 3H), 7.85 (t, 1H, *J* 6.9 Hz), 7.76-7.65 (m, 3H).

Entry 7: *N'-((2-Chloroquinolin-3-yl) methylene) benzohydrazide*: Color White, Yield of 83%, mp 208–209 °C.

FT-IR (KBr) νmax/cm^{-1}: 3748, 3182, 2923, 1698, 1618, 1600, 1563. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.30 (s, 1H, NH), 8.98 (s, 1H), 8.97 (s, 1H), 8.25 (d, 1H, *J* 8.1 Hz), 7.98 (m, 3H), 7.86 (m, 1H), 7.74-7.57 (m, 4H).

Entry 8: *N'-((2-Chloroquinolin-3-yl) methylene)-2-phenoxyacetohydrazide*: Color Off white, Yield of 95%, mp 169–170 °C.

FT-IR (KBr) νmax/cm^{-1}: 3279, 3194, 2939, 1681, 1614, 1597, 1585. ^{1}H NMR (DMSO-d_6, 400 MHz) δ:12.10 (s, 1H, NH), 9.02 (s, 1H), 8.85 (s, 1H), 8.22 (m, 1H), 7.98 (d, 1H, *J* 8 Hz), 7.84 (m, 1H), 7.70 (m, 1H), 7.32 (m, 2H), 7.00 (m, 3H), 5.23 (s, 2H).

Bacterial Isolates

The antibacterial activities of the benzohydrazide derivatives were examined against four Gram-positive bacteria, namely, *S. aureus*, *E. faecalis, Bacillus subtilis*, and diphtheroids, as well as five gram-negative bacteria including *E. coli, K. pneumoniae, P. aeruginosa, S. marcescens*, and *S. enterica.* The bacteria were obtained from clinical samples, propagated on nutrient agar (Merck, Germany) at 37°C, and maintained at 4°C until use.

In Vitro Antibacterial Assessment

In this study, the antibacterial activities of the benzohydrazide derivatives were assessed by the agar well-diffusion method. A suspension containing 1.5×10^8 CFU/mL bacteria in sterile normal saline (adjusted to 0.5 McFarland standard) was initially prepared (26). Muller-Hinton agar (Merck, Company) with a depth of 4 mm was poured into petri dishes to give a solid plate. Next, 100 µL of the bacterial suspension was inoculated into the medium by sterile cotton swabs. Afterward, 6-mm diameter punctures were created within the culture media by sterile cork borers and filled with 20 µL of each benzohydrazide derivative. The first used concentration was 80 mg/mL, and the plates were incubated at 37°C for 24 hours. Following incubation, the antibacterial activity was determined by measuring the zones of inhibition (mm) around each well. All the tests were performed in triplicate. DMSO: methanol (1:1 v/v) solvent and tetracycline were considered as negative and positive controls, respectively. To determine minimum inhibitory concentration (MIC), a two-fold dilution series (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, and 0.3 mg/mL) of each benzohydrazide derivative was prepared in DMSO: methanol (1:1 v/v) solvent and bioassayed using the agar well-diffusion method as mentioned above. The cultures were then incubated at 37°C for 24 hours (27).

Results

In our ongoing search for bioactive substances and in connection with our efforts to synthesize benzohydrazide derivatives, we here investigated the antibacterial activities of these chemical adducts. First, we described an efficient protocol for preparing these compounds. The results of antimicrobial tests showed that these derivatives were active against *S. aureus*, *E. coli*, *E. faecalis*, *B. subtilis*, diphtheroids, *S. enterica*, *S. marcescens*, *P. aeruginosa*, and *K. pneumoniae.*

Initially, we examined the efficiency of the synthesis process of N'-((2-Chloroquinolin-3-yl) methylene)-2-methoxybenzohydrazide. To this end, 2-methoxybenzhydrazide (0.332 g, 2 mmol) and 2-chloroquinoline-3-carbaldehyde (0.383 g, 2 mmol) were mixed and ground in a pestle. The mixed powder was transferred to a round-bottomed flask containing the green catalyst liquid. Overall, we here presented a simple method for synthesizing benzohydrazide derivatives using the green catalyst liquid as an eco-friendly, inexpensive, and efficient reagent. Short reaction times, high yield, the simplicity of the process, and an easy work-up procedure are some of the advantages of this method. Our promising results suggest the necessity of evaluating the antibacterial activities of other structural derivatives of *benzohydrazide*. The results of FT-IR and ^{1}H NMR analyses of N'-((2-Chloroquinolin-3-yl) methylene)-2-methoxybenzohydrazide have been shown in Figures 1 and 2.

According to the antibiogram test (i.e. agar well-diffusion assay), all of the bacterial isolates were sensitive to the used benzohydrazide derivatives at 80 mg/mL concentration, delivering inhibition zones ranging from 11 to 42 mm (Table 1). The MIC values of benzohydrazide derivatives have been shown in Table 2.

Discussion

Increased resistance to antibiotics is an alarming health threat as treating the infections caused by resistant bacterial strains can be problematic (28). This study aimed to examine the antibacterial activities of benzohydrazide

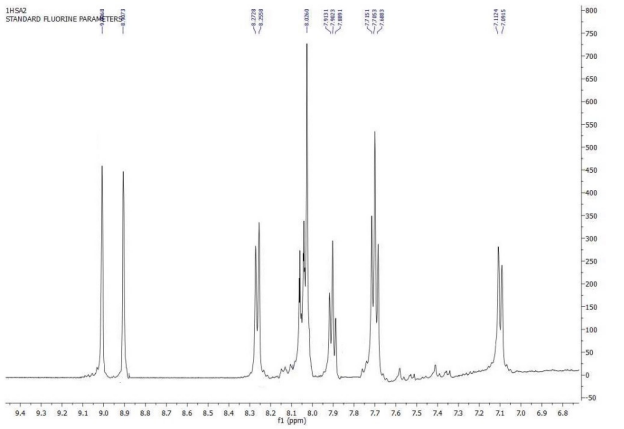

Figure 1. Expanded ^{1}H NMR Analyses of N'-((2-Chloroquinolin-3-yl) Methylene)-2-Methoxybenzohydrazide.

Table 1.Inhibition Zones (mm) of Eight Benzohydrazide Derivatives at 80 mg/mL Concentration Against Nine Bacteria as Assessed by Agar Well-diffusion Assay

Bacteria	Derivatives							
	4-Chloro-N′-((2-chloroquinolin-3-yl)methylene) benzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-methoxybenzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2,4-dinitrobenzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-phenoxyacetohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-(3,5-dichlorophenoxy) acetohydrazide	N′-((2-Chloroquinolin-3-yl) methylene)-4-nitrobenzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene) benzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-hydroxybenzohydrazide
S. aureus	-	12	15	15	20	23	23	17
E. faecalis	11	16	10	24	25	26	25	15
B. subtilis	-	13	20	17	25	17	25	20
Diphtheroids	12	-	25	40	40	34	34	42
E. coli	12	20	19	18	22	12	18	20
S. enterica	11	17	12	24	21	15	22	25
S. marcescens	12	20	16	23	23	14	21	22
P. aeruginosa	11	15	16	13	24	11	18	20
K. pneumoniae	11	21	18	26	21	13	21	26

Table 2. MIC (mg/mL) Values of Eight Benzohydrazide Derivatives Against Nine Bacteria as Determined by Agar Well-diffusion Assay

Bacteria	Derivatives							
	4-Chloro-N′-((2-chloroquinolin-3-yl)methylene) benzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-methoxybenzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2,4-dinitrobenzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-phenoxyacetohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-(3,5-dichlorophenoxy) acetohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-4-nitrobenzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene) benzohydrazide	N′-((2-Chloroquinolin-3-yl)methylene)-2-hydroxybenzohydrazide
S. aureus	aa	20	40	5	20	20	10	10
E. faecalis	40	20	2.5	5	10	2.5	10	20
B. subtilis	-	10	10	2.5	0.625	0.625	10	10
Diphtheroids	0.625	10	10	0.625	0.625	0.625	2.5	10
E. coli	40	20	40	10	0.625	10	2.5	20
S. enterica	20	10	40	10	10	1.25	5	0.625
S. marcescens	40	10	2.5	5	0.625	1.25	10	20
P. aeruginosa	40	10	5	0.625	0.625	5	20	10
K. pneumoniae	40	20	40	10	10	80	10	20

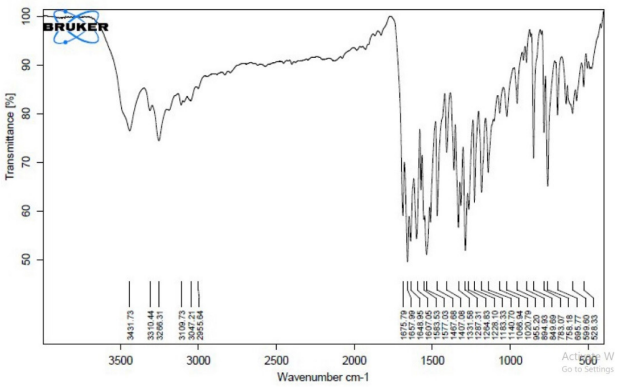

Figure 2. FT-IR Analyses of N′-((2-Chloroquinolin-3-yl) Methylene)-2-Methoxybenzohydrazide.

derivatives against some pathogenic bacteria using *in vitro* assays. To achieve this goal, we challenged nine bacterial isolates with different concentrations of benzohydrazide derivatives. Based on the obtained results, benzohydrazide derivatives showed significant antibacterial activities against the evaluated bacteria. Similar studies have investigated the antimicrobial effects of some biologically active chemical reagents. In one study, a synthetic series of [3/4-bromo-N&apos-(substituted benzylidene/furan-2-ylmethylene/5-oxopentylidene/3-phenylallylidene), benzohydrazides (1-23)] were subjected to physicochemical and spectral characterization. The synthesized compounds were also screened for their antimicrobial and anticancer features. Antimicrobial tests indicated that one of the compounds (number 12, pMICam = 1.67 µM/mL) delivered the most potent antimicrobial activity (29). Furthermore, in another study, 14 derivatives of 4-substituted [*N*′-(benzofuroxan-5-yl) methylene] benzohydrazides nifuroxazide were synthesized and tested against the standard multidrug-resistant *S. aureus* strains. Twelve out of the 14 compounds exhibited bacteriostatic activities against the evaluated strains (30). In the study of Ghasemi et al, thiazole derivatives significantly inhibited the growth of *B. cereus* and *L. monocytogenes,* but not *E. coli* and *S. typhimurium* (31). Setyawati et al also synthesized chalcone-like benzohydrazide derivatives applying natural vanillin and wintergreen oil and investigated their potential as natural antimicrobial agents. The products were analyzed by FT-IR, GCMS, 1H- and 13C-NMR to confirm their structure, and they were also slightly modified to increase their biological activities. In a recent study, 5-bromovanillin, salicyl hydrazine, and benzohydrazide were successfully synthesized with the yields of 98%, 78%, and 33%, respectively. These compounds also showed the best antimicrobial activities against Gram-positive and Gram-negative bacteria (32).

Conclusions

Our findings revealed the potential of benzohydrazide derivatives as potent antibacterial agents. Hence, these compounds can be exploited as antiseptic coatings for materials surfaces and for a variety of other environmental and biomedical applications.

Conflict of Interests

Authors have no conflict of interests to declare.

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Authors' Contribution

We confirm that the manuscript, as well as the order of authors listed in the manuscript, has been contributed, reviewed and approved by all named authors.

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