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Synthesis, Structure Elucidation and Biological Activity of New Hybrid Hydrazone-Amide Compounds

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ABSTRACT

Bacterial infection today occupies a tremendous place in world health. The infection diseases were kept under control after the development of penicillin and further studies were performed on the development of new antibacterial agents. However, to date, bacterial resistance caused a big failure in the treatment of infectious disease and therefore, development of new antibacterial agents became important for human health. In the present study, we have designed, synthesized and elucidated the structures of new hydrazide-hydrazone compounds and their hybrid amide derivatives. The structures of the compounds were elucidated with spectroscopic methods and their purity were proven by TLC, HPLC-MS analysis. The antibacterial and antifungal activity studies of the novel molecules were investigated on different strains. Among the synthesized compounds, AA3a and AA4a appeared to show promising antibacterial activity. None of the compounds showed significant antifungal activity on *Candida albicans*. The drug likeness properties and boiled-egg plot analysis were performed for all of the compounds. The novel molecules showed no violation on Lipinski's rule of five and all the molecules showed good gastrointestinal absorption properties in the *in silico* studies.

Keywords: 4-ASA, Hydrazide, Hydrazone, Amide, Antibacterial activity.

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Introduction

Infectious diseases have been a tremendous threat to human health for centuries. First success was achieved via the discovery of penicillin and bacterial infections were kept in control over a period of time. To date, many new antibiotics were presented to human health services but the treatment failed due to the low bioavailability, adverse effects and uncontrolled release of these compounds [1]. Over the past decades, unfortunately bacteria showed resistance to current antibiotics with a fast adaptation to the environment. For a certain period of time, discovery of new bacterial targets resulted with the development of more effective antibiotics; however, scientists still face with different bacterial resistances [2]. In order to overcome the resistance, bacterial life circle must be studied in detail. A new bacterial macromolecule could be an answer to both bacterial resistance and infectious disease treatment. The design of a new drug passes through a series of process that involved in medicinal chemistry [3-5]. The drug spectrum mainly identifies with MIC dilution assays, which most likely provide a detailed insight into the drug. Besides the MIC studies, *in silico* techniques also support and shorten the discovery time of the drug candidate [6].

Medicinal chemistry studies include a series of steps starting with the identification of pharmacophore groups. The effects of many heterocyclic rings and different functional groups have been studied over a period of time. Among them, studies on hydrazide-hydrazone structures occupies a vital place in drug discovery process. Hydrazones consist of a structure of -HC=N-NH_2. However, hydrazide-hydrazones have -HC=N-NH-CO- structure, an additional carbonyl group. The synthetic route for hydrazide-hydrazones involves a reaction between hydrazide and substituted aldehyde/ketone. The reaction sometimes requires no catalyst; but in some circumstances, an acid may facilitate the reaction. Their synthetic procedure requires no complex conditions; therefore, these class of compounds are extensively studied over a period of time. Besides their high yield synthetic route, they possess diverse uses such as anticancer, antimicrobial, anticonvulsant, antidiabetic, anti-tuberculosis, antitumor, antidepressant, anti-inflammatory, and antiviral activities [7]. Metabolically, hydrazide-hydrazones cause a lower toxicity than hydrazides since the free amino group turns into an azomethine moiety. The *in vitro* metabolic studies also showed the hydrolytic profile of hydrazide-hydrazone structures [8, 9]. The specific physicochemical properties of the hydrazide-hydrazones made them an important functional group on antibacterial drug development, which may eliminate the microbial resistance to the current drugs [10]. Nifuroxazide is one of the leading molecule that contains a hydrazide-hydrazone functionality and it exerts antiseptic effects. Mitoguazone (anticancer), ferimzone (fungicide), dihydralazine (antihypertensive) have hydrazone functionality. We,

therefore, decided to design a series of hydrazone containing molecules at the first place.

The dual activities or more pharmacophore group has advantages on the biological activities of drugs. The design of our study started with hydrazide-hydrazone synthesis. We have included one more step for our compounds by introducing amide, another important functional group which is available in the structure of antibacterial, local anaesthetic, analgesic and anticancer drugs.

In the light of the foregoing, we have designed and synthesized a series of novel hydrazide-hydrazone compounds starting from 4-aminosalicylic acid (4-ASA) as amide hybrids. We have elucidated their structure with spectroscopic methods and purified them with column chromatography; afterwards the purity of the compounds were elucidated by LC-MS studies. Their antibacterial activities were investigated on different bacterial strains. Among the synthesized compounds, compound AA3a (hydrazide-hydrazone) and AA4a (hydrazide-hydrazone/amide hybrid) showed promising antibacterial activity.

Materials and Methods

General

All the chemicals were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO). Reactions were monitored by TLC on silica gel plates purchased from Merck (Merck Co., Darmstadt, Germany). Melting points of the synthesized compounds were determined in a Stuart SMP50 Automatic Melting Point apparatus and these are uncorrected. The purity of the compounds was confirmed by TLC, LC-MS. NMR spectra were recorded on BRUKER 400 MHz (Billerica, MA) for ^{1}H-NMR. Data are reported as follows: chemical shift, multiplicity (b.s.: broad singlet, d: doublet; m: multiplet, s: singlet, and t: triplet), coupling constants (Hz), integration. An Agilent 1260 Infinity II HPLC-MS spectra equipped with G7114A 1260DAD detector, G7311B 1260 Quad Pump system, G1328C 1260 manual injection unit and G6125B LC/MSD detector was used for both HPLC and mass analysis. Retention times were recorded with ACE C18 column (particle size: 3 µm, pore size: 100A). The column temperature was adjusted to 25^{o}C in the column compartment. The mobile phase consisted of acetonitrile-water (80:20, v/v) mixture and delivered at a flow rate of 0.8 ml/min. The injection volume was 20 µL. The UV detector was operated at 254 nm. R_fx100 values were recorded on petroleum ether/ethyl acetate/gl. acetic acid (different ratios, see Table 2). Mass spectral analysis were performed with Advion Expression CMS device, ASAP probe, Advion Chem express software. Samples were scanned as positive and negative ion and were directly applied into the device via an ASAP probe.

Synthetic procedure for methyl 4-amino-2-hydroxybenzoate (AA1)

4-aminosalicylic acid (0.01 mol) was dissolved in methanol (30 ml) and few drops concentrated sulphuric acid was added. The mixture was heated under reflux for 20-24 h and the reaction was monitored with TLC. After the reaction was completed, the mixture was neutralized with 10% $NaHCO_3$ and the solid obtained was filtered and recrystallized with ethanol [11].

Synthetic procedure for 4-amino-2-hydroxybenzohydrazide (AA2)

4-ASA ester (AA1) (0.01 mole) was dissolved in ethanol (10 ml) and excess hydrazine hydrate was added. The reaction was refluxed for 4-6 hours and monitored with TLC. After the reaction was completed, the mixture was evaporated under atmospheric pressure. The crude product was recrystallized with ethanol. [12]

General procedure for the synthesis of compounds 4-amino-N'-[(E)-(4-substitutedphenyl)methylidene]-2- hydroxybenzohydrazide (AA3a-b)

4-ASA hydrazide (AA2) (0.001 mol) was dissolved in ethanol and equimolar amount of substituted benzaldehyde was added in the presence of few drops concentrated hydrochloric acid. The reaction mixture was refluxed for 6 hours and monitored with TLC. After the reaction was completed, ice-cold water was added and precipitate formed was filtered, dried and purified with column chromatography.

Synthetic procedure for N-(4-{[(2Z)-2-(4-chlorobenzylidene) hydrazinyl]carbonyl}-3- hydroxyphenyl)benzamide (AA4a)

Compound AA3a (0.001 mole) was dissolved in THF and sodium carbonate (30 mg) was added. Benzoyl chloride (0.003 mole) was added dropwise in cold and the reaction mixture was kept in room temperature for 4 hours. The reaction was monitored with TLC. After the reaction was completed, the precipitate was filtered and purified with column chromatography.

Methyl 4-amino-2-hydroxybenzoate (AA1)

Gray crystal. Yield 91 %; m. p. 121°C; MW: 167.05 g/mol; Rfx100 value: 74.6; Rt value: 12.12 min. ^{1}H-NMR (400 MHz) (DMSO-d_6/TMS) δ ppm: 10.78 (s, 1H, Ar-OH), 7.46 (bs, 2H, Ar-NH_2), 6.14-6.00 (m, 3H, Ar-H), 3.79 (s, 3H, $COOCH_3$). MS (vAPCI): [M^+1]: 168.

4-Amino-2-hydroxybenzohydrazide (AA2)

Orange solid. Yield 98 %; m. p. 197°C; MW: 167.06 g/mol; Rfx100 value: 4.7; Rt value: 6.40 min. ^{1}H-NMR (400 MHz) (DMSO-d_6/TMS) δ ppm: 12.45 (bs, 1H, NH-NH_2), 9.51 (s, 1H, Ar-OH), 7.43 (bs, 2H, Ar-NH_2), 6.18-5.70 (m, 3H, Ar-H), 4.39 (bs, 2H, NH-NH_2). MS (vAPCI): [M+1]: 168.

4-Amino-N'-[(E)-(4-chlorophenyl)methylidene]-2-hydroxybenzohydrazide (AA3a)

White solid. Yield 97 %; m. p. 250-252°C; MW: 289.06 g/mol; Rfx100 value: 62.2; Rt value: 19.20 min. ^{1}H-NMR

(400 MHz) (DMSO-d_6/TMS) δ ppm: 12.43 (bs, 1H, -NH-N=CH-), 11,60 (s, 1H, -NH-N=CH-), 8.39 (s, 1H, Ar-OH), 7.75-5.49 (m, 8 H, Ar-H and *Ar-NH_2). MS (vAPCI): [M+1]: 290. **Ar-NH_2 was exchanged with deuterium*

4-Amino-N'-[(E)-(4-bromophenyl)methylidene]-2-hydroxybenzohydrazide *(AA3b)*

Yellow solid. Yield 75%; m. p. 240-242 °C; MW: 333.01 g/mol; Rfx100 value: 63.1; Rt value: 14.01 min. ^{1}H-NMR (400 MHz) (DMSO-d_6/TMS) δ ppm: 12.39 (bs, 1H, -NH-N=CH-), 11,80 (s, 1H, -NH-N=CH-), 8.39 (s, 1H, Ar-OH), 7.87-5.93 (m, 8 H, Ar-H and *Ar-NH2). MS (vAPCI): [M+1]: 334. **Ar-NH_2 was exchanged with deuterium*

N-(4-{[(2Z)-2-(4-chlorobenzylidene) hydrazinyl]carbonyl}-3-hydroxyphenyl)benzamide *(AA4a)*

Yellow solid. Yield 80.05 %; m. p. 235-237°C; MW: 393.08 g/mol; Rfx100 value: 65; Rt value: 9.12 min. ^{1}H-NMR (400 MHz) (DMSO-d_6/TMS) δ ppm: 12.97 (bs, 0.3H, CONH), 12.31 (s, 1H, Ar-OH), 10.80-10.55 (m, 2H, -NH-N=CH-), 8.16-6.87 (m, 12H, Ar-H). MS (vAPCI): [M+1]: 394.

Antimicrobial Activity Test

The six compounds were tested for their antimicrobial activities against *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 (Gram-positive bacteria), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative bacteria) and *Candida albicans* ATCC 10231 (fungus). This test was performed according to the Clinical Laboratory Standards Institute (CLSI) M100-S28 protocol for bacteria [10] and CLSI M27-A3 protocol for fungi [14]. Mueller Hinton Broth (MHB) and RPMI-1640 mediums were used for determination of antibacterial and antifungal activity of the compounds, respectively.

The compounds were dissolved in 10% DMSO. The serial dilutions of each compound at the range of 512-2 µg/mL were prepared in 96-well microplates, after placing broth mediums in each well. Suspension of each microorganism was prepared using McFarland 0.5 standard and as a result 10^5 cfu/ml densities were reached. Microplates were incubated for 24 hours at 37°C for bacteria and for 48 hours at 35°C for fungus. The reference drugs were tested against these microorganisms. Besides, growth control of microorganisms and sterilization control of the mediums were tested. 10% DMSO as solvent in this study was tested for its potential antimicrobial activity. The wells with the lowest concentration without microbial growth were determined as minimum inhibition concentration (MIC). The detection was made by visual evaluation using dye MTT [15]. The test repeated 3 times.

In Silico Properties

Drug likeness properties were studied using SwissADME program. The SMILE codes of the compounds were inserted and the ADME predictions were run via the program. The results displayed in Table 1.

Table 1. Some properties of synthesized compounds from SwissADME

	HBA	HBD	TPSA	Log $P_{o/w}$	SC	-Log K_p	GIA	RoF (V)	Ghose	Leadlikeness (V)
4-ASA	3	3	83.65	0.84	Soluble	6.30	High	0	No	No
AA 1	3	2	72.55	1.31	Soluble	6.15	High	0	Yes	No
AA 2	3	4	101.37	0.82	Soluble	7.09	High	0	No	No
AA 3a	3	3	87.71	1.97	Moderate	5.96	High	0	Yes	Yes
AA 3b	3	3	87.71	1.80	Moderate	6.18	High	0	Yes	Yes
AA 4a	4	3	90.79	2.70	Poor	5.18	High	0	Yes	No

MW: Molecular weight, **HBA:** H-bond acceptor, **HBD:** H-bond donor, **TPSA:** Topologic polar surface area (Å^2) **Log $P_{o/w}$:** *Consensus* Log $P_{o/w}$ (Average of all five predictions), **SC:** Solubility Class (Water), **GIA:** Gastrointestinal absorption, **Log K_p:** skin permeation **(-cm/s)**, **RoF (V)**: Rule of Five (violation number), **Ghose:** Ghose Filter, **Leadlikeness (V):** Suitability score (violation number).

Results and Discussion

Chemistry

4-Aminosalicylic acid (4-ASA) was chosen as a starting compound for this study. Its ester and hydrazide derivatives were synthesized accordingly; confirming with the literature data [11, 12]. The corresponding hydrazide derivatives were then converted into their hydrazide-hydrazone structures. Briefly, 4-ASA hydrazide was treated with substituted benzaldehydes in the presence of hydrochloric acid. The hydrazide-hydrazones are generally known to be produced in high yield with no catalyst needed. First attempts for hydrazone synthesis were done only in room temperature. However, the reaction only proceeded in the presence of concentrated hydrochloric acid. Although, there are several solvents available for hydrazone synthesis, we decided to perform the synthesis in absolute ethanol as it has less toxicity than other solvents. The amide formation of the hydrazone structures were tricky. The first attempt was to add benzoyl moiety in the beginning of the synthesis. However, when doing so, the amide was hydrolyzed easily in the esterification process. Furthermore, benzoylation of hydroxyl group could be another problem for the following steps. Eventually, we decided to synthesize amide in the last step. The formation of amide was

performed using Schotten Baumann reaction. For this, hydrazide-hydrazone was dissolved in tetrahydrofuran (THF) and small amount of sodium carbonate was added to provide basic conditions. The benzoyl chloride was added dropwise and the reaction was kept in room temperature for several hours. The chlorine substituted derivative underwent amide formation but the reaction could not be completed in the case of bromine substituted derivative. It was assumed that the steric hindrance resulting from bromine could be the main cause (Figure 2). All the reaction processes were monitored by thin layer chromatography (TLC) with the mobile phases listed below (Table 2). The compounds were purified with crystallization and column chromatography. The purity of the compounds were also confirmed by LC-MS.

Table 2. TLC mobile phase conditions

Compound	Mobile Phase	Rfx100 Value
AA1	S1: Ethyl acetate:petroleum ether +100µl g. acetic acid (5/5, v/v)	AA1:74.6
AA2		AA2: 4.7
AA3a-b	S2: Ethyl acetate:petroleum ether +100µl g. acetic acid (7/3, v/v)	AA3a:62.2 AA3b:63.1
AA4a	S3: Ethyl acetate:petroleum ether +100µl g. acetic acid (6/4, v/v)	AA4a:65

The structures of original hydrazide-hydrazone and amide hybrids were elucidated by ^{1}H-NMR and MS spectral techniques. The results proved the proposed structures of the compounds. ^{1}H-NMR spectra showed the formation of ester and hydrazide derivatives from 4-ASA. Methyl protons of AA1 were recorded at 3.79 ppm with 3 integration values. The disappearance of carboxylic acid proton was also another proof for the presence of ester. The hydrazide formation was confirmed by observing the presence of –CO-NH-NH_2 protons. The methyl protons of AA1 disappeared and two broad singlet peaks were recorded at 7.43 and 4.39 ppm respectively. As -NH- proton is close to carbonyl group, it shifted down field and recorded at 7.43 ppm, 1H integration. -NH_2 protons were recorded at 4.39 ppm, 2H integration. The formation of hydrazide-hydrazone was monitored with azomethine proton in NMR spectra. The ^{1}H-NMR results indicated the formation of hydrazide-hydrazone with the disappearance of NH_2 protons from compound AA2. The peak recorded at 4.39 ppm disappeared and singlet peaks were recorded at 11.60-11.80 ppm for AA3a and AA3b respectively. However, aromatic amine protons were exchanged with deuterium as this is a common condition for protons bonded with heteroatoms. Same situation was observed for AA4a, as the amide proton was recorded as 0.3 H integration value. The chemical shift for this proton was strong because of carbonyl and aromatic rings. Therefore, the formation of the amide hybrid was proved by a peak recorded at 12.97 ppm.

For all the synthesized compounds, mass spectral analysis were performed both with LC-MS and Mass Spectra (Volatile Atmospheric Pressure Chemical Ionization (vAPCI). The molecular ion peaks were recorded as calculated (M+1) (See Supplementary file).

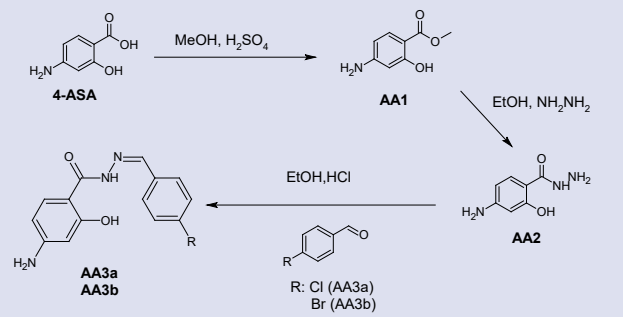

Figure 1. Synthetic route to hydrazide-hydrazone compounds

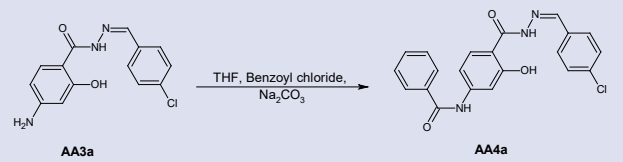

Figure 2. Synthetic route to hydrazide-hydrazone amide hybrid

Biological Activity

Several studies were reported for the biological activities of hydrazide-hydrazone structures [16-35]. The hydrazone group has an advantage of blocking the free amine for metabolic stability. Thus, the potential toxicity of the hydrazones could be compared to hydrazone derivatives. We therefore decided to study their antibacterial and antifungal activities on different bacterial and fungal strains. The MIC values were determined for each compound and ampicillin, gentamicin, vancomycin for antibacterial and fluconazole for antifungal activity were used as reference drugs. The results were listed in Table 3.

The antibacterial results obtained in the present study gave a general overview for certain functional groups of the synthesized compounds. It was understood from the results that compounds have moderate antimicrobial activity to both Gram positive and Gram negative bacteria. All of the synthesized compounds and 4-ASA showed weak activity to *S. aureus* ATCC 29213 with MIC value of 256 µg/ml. Thus, the modifications on the functional groups caused no difference for *S. aureus* strain. On the other hand, compounds showed promising activity against *E. faecalis* ATCC 29212 with the MIC value ranging from 64-256 µg/ml. Introduction of carboxylic acid, ester and hydrazide groups showed no difference in the activity against *E. faecalis*. However, chlorine substitution of hydrazide-hydrazone made a difference on the activity. This could be due to proper lipophilicity of the compound. When chlorine is replaced with bromine atom, the activity decreases. As bromine atom is larger than chlorine, the bromine derivative might have difficulty in reaching cellular targets. Besides, amide hybrid altered the activity profile in a negative way. With those results, we can conclude that larger molecules may lower the antibacterial activity on Gram-positive bacteria.

For Gram-negative bacterial strains, compounds showed weak antibacterial activity (except for compound AA4a). The functional group modifications form carboxylic acid to ester, hydrazide and hydrazide-hydrazone favoured the antibacterial activity. There is also a decrease on the antibacterial activity of compound AA3b, which has a bromine atom, on *E. coli* strain. This could also be a result of the bulky bromine group. On the contrary, the hybrid molecule showed the best antibacterial activity on *P. aeruginosa* strains.

It was assumed that the peptidoglycan barrier made no significant difference in terms of antibacterial activity. In a way, the target macromolecule of the compounds could not be enzymes in peptidoglycan biosynthesis. Because, cell wall difference made no change in the antibacterial activity. This could partly explain the small difference between Gram-positive and Gram-negative antibacterial activity. However, in both cases, hydrazine and amide functional groups caused dramatic increases in the activity. It is difficult to analyse the structure-activity relationship with few substitutional changes but the bulky group made a notable decrease in the activity on bacterial strains.

The diverse biological activities of hydrazone compounds led us to investigate the *C. albicans* strains. In our present study, there are no significant antifungal activity detected for all of the compounds. This could be the result of no interaction with lanesterol 14α-demethylase enzyme in ergosterol synthesis. Thus, the MIC values of all compounds on *C. albicans* ATCC 10231 were at the range of 128-512 µg/ml.

Table 3: *In vitro* MICs (µg/mL) observed of the compounds and reference antimicrobial drugs

COMPOUNDS		MICROORGANISMS				
		S. a.	E.f.	E. c.	P.a.	C. a.
1	4-ASA	256	128	256	256	128
2	AA-1	256	128	128	128	256
3	AA-2	256	128	128	128	>512
4	AA-3a	256	64	128	128	128
5	AA-3b	256	128	256	128	128
6	AA-4a	256	256	256	64	256
	Ampicilin	2	2	16	NT	NT
	Gentamycin	1	2	1	1	-
	Vancomycin	1	2	NT	NT	NT
	Fluconazole	NT	NT	NT	NT	1

S.a.: *Staphylococcus aureus* ATCC 29213; E.f.: *Enterococcus faecalis* ATCC 29212; E.c.: *E. coli* ATCC 25922; P.a.: *Pseudomonas aeruginosa* ATCC 27853; C.a: *Candida albicans* ATCC 10231
NT: Not Tested

Drug Likeness Properties

A SwissADME program was used for calculation of physicochemical properties of the synthesized compounds and 4-ASA [36]. The detailes were listed in Table 1. The parameters that are most likely to be considered in drug development were calculated virtually. H-bond acceptor (HBA), H-bond acceptor (HBD), polar surface area (PSA/TPSA), partition coefficient (Log Po/w), skin permeability (Log Kp), Rule of five (violation number-RoF) and Gastrointestinal absorption (GIA) were calculated and evaluated for the suitability. According the Lipinski's rule of five, all of the compounds showed no violation and presented to have good bioavailability. All of the novel molecules passed the Ghose filter. However, lead likeness were applicable interestingly only for hydrazide-hydrazone compounds. The boiled-egg plot was also investigated for the absorption of compounds from gastrointestinal system and penetration of them from the brain. All of the compounds showed good absorption on gastrointestinal tract but their brain permeability was poor (Figure 3).

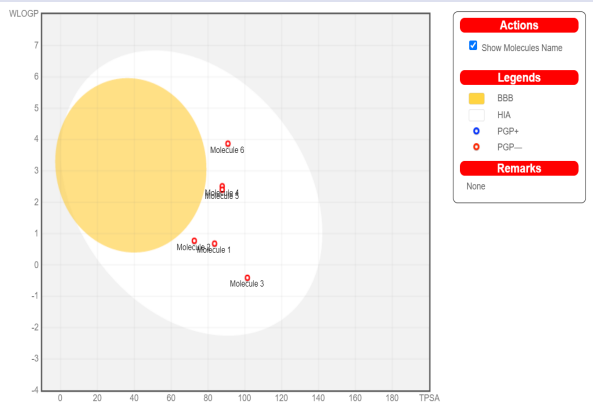

Figure 3 Molecule 1: 4-ASA, molecule 2: 4-ASA ester, molecule 3: 4-ASA hydrazide, molecule 4:bromo substituted hydrazinde-hydrazone, molecule 5: chloro substituted hydrazide-hydrazone, molecule 6: hydrazide-hydrazone/amide hybrid

Conclusion

In the present study, we have synthesized and elucidated the structures of novel hydrazide-hydrazone compounds and a hydrazide-hydrazone/amide hybrid drug candidate. The structures of the compounds were elucidated using spectroscopic methods and purity of the compounds were confirmed by chromatographic methods. The compounds having hydrazide-hydrazone and the amide hybrid showed promising antibacterial activity on tested bacterial strains. The antibacterial mechanism of action could possibly be depending on the physiochemical properties of the compounds. Further studies should be carried out in order to find out the correct mechanism of the new lead molecules.

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Conflicts of interest

There are no conflicts of interest in this work.

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