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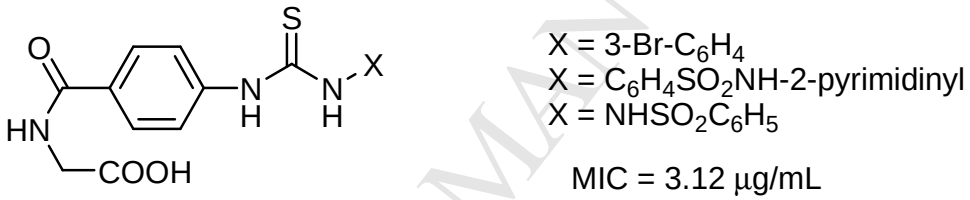
Graphical abstract:



New series of thiourea derivatives were synthesized through reaction with 4-hippuric acid isothiocyanate and evaluated for antibacterial and antifungal activity.

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Thiourea derivatives incorporating a hippuric acid moiety: Synthesis and evaluation of antibacterial and antifungal activities

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Abstract: New series of thiourea derivatives incorporating a hippuric acid moiety have been synthesized through the reaction of 4-hippuric acid isothiocyanate with various nitrogen nucleophiles such as aliphatic amines, aromatic amines, sulfa drugs, aminopyrazoles, phenylhydrazine and hydrazides. The synthesized compounds were tested against bacterial and fungal strains. Most of compounds, such as 2-(4-(3-(3-bromophenyl)thioureido)benzamido)acetic acid and 2-(4-(3-(4-(*N*-pyrimidin-2-ylsulfamoyl)phenyl)thioureido)benzamido)acetic acid, showed significant antibacterial and antifungal activities. These compounds comprise a new class of promising broad-spectrum antibacterial and antifungal agents.

Keywords: Thiourea; Hippuric acid; Antibacterial; Antifungal activity.

1. Introduction

Despite many significant progresses in antimicrobial therapy, infectious diseases caused by bacteria and fungi remain a major worldwide health problem due to rapid development of resistance to the existing antimicrobial drugs. In other words, the increasing use and misuse of the existing antimicrobial drugs have resulted in the development of resistant pathogens [1-4]. So, the medical community faces a serious problem against infections caused by the pathogen microbes and needs an effective therapy and search for novel antimicrobial agents. The emergence of multidrug resistant gram-positive and gram-negative bacteria has caused life-threatening infectious diseases in many countries around the world. In addition, systemic and dermal fungal infections have significantly increased, specifically in individuals with suppressed immune systems such as cancer chemotherapy and AIDS patients. Although there are different antifungal drugs used in the treatment of fungal infections, some of them have undesirable side effects because of the biochemical similarity to human cell. Moreover, some of them become less effective due to the development of

resistance to these drugs. The search for new and effective antimicrobial agents, resistant to the mechanisms of defense of these bacteria, is of paramount importance [5, 6].

Various 1,3-disubstitued thiourea derivatives are extremely versatile building blocks for the synthesis of a variety of heterocyclic compounds and exhibit a wide spectrum of bioactivities. Many thiourea derivatives showed significant antimicrobial activity [7]; 1,3-dialkyl or diaryl thiourea derivatives exhibit significant antifungal activity against plant pathogens [8].

As a part of our extensive research program to rapidly assemble novel bioactive compounds under mild conditions [9-13], we have employed 4-hippuric acid isothiocyanate to synthesize various 1,3-disubstituted thiourea derivatives with structure modifications involving incorporation of the hippuric acid moiety at position N^1 with promising antimicrobial properties.

2. Chemistry

The monosubstituted thiourea **2** was obtained through the reaction of 4-aminohippuric acid **1** with ammonium thiocyanate in the presence of hydrochloric acid. The interest of 4-aminohippuric acid **1** is based on the conversion possibility of the amino group in position 4 to isothiocyanate group. The isothiocyanate **3** was synthesized from the reaction of 4-aminohippuric acid **1** with thiophosgene in the presence of dilute HCl at room temperature. The scope of the reaction of isothiocyanate **3** with various nitrogen nucleophiles was studied with the objective of obtaining biologically active compounds. When isothiocyanate **3** was left to react with some selected aliphatic and aromatic amines, the corresponding thiourea derivatives **4a-g** were obtained in good yield. Supportive evidence the latter products was obtained from their synthesis *via* another synthetic route. Thus, interaction of the amine **1** with isothiocyanate derivatives in dioxane gave the thiourea derivatives **4a-g** (m.p. and mixed m.p.) (Scheme 1).

Scheme 1

Moreover, our investigation was extended to include the behavior of isothiocyanate **3** towards different types of aromatic amines. Thus, interaction of **3** with 9-aminoacridine as polynuclear aromatic amine gave the corresponding thiourea derivative **5**. Interaction of **3** with 2-amino-*N*-(aryl)-benzamides gave the corresponding benzamide thiourea derivatives **6a,b**. It's well known that sulfonamides have enormous potential as pharmaceutical and agricultural agents due to their biological activities; they are associated with antibacterial properties. Some active sulfonamides as antibacterial agents are also known for their immune-modifying effects. On the basis of these reports, we reported here the synthesis of thiourea derivatives containing sulfonamide moiety to evaluate their antimicrobial activity. Thus, interaction of **3** with some selected sulfa drugs gave the corresponding thiourea derivatives **7a-f** (Scheme 2). Symmetrical bis-hippuric acid **8a** was obtained when one mole

of compound **1** was treated with one mole of isothiocyanate **3** in dioxane under reflux. On the other hand, symmetrical bis-compound **8b** was obtained when two moles of compound **3** were reacted with one mole of 1,4-phenylenediamine in dioxane under reflux (Scheme 2).

Scheme 2

The pyrazole derivative **9** was obtained *via* the reaction of the isothiocyanate **3** with 4-aminoantipyrene. Treatment of **3** with 5-amino-3-phenylamino-1*H*-pyrazole-4-carboxylic acid amide as aminopyrazole afforded the corresponding pyrazole derivative **10a.** Moreover, treatment of **3** with 4-(4-chloro-phenylazo)-4*H*-pyrazole-3,5-diamine afforded the corresponding pyrazole derivative **10b (**Scheme 3). Treatment of **3** with phenylhydrazine in dioxane furnished the novel disubstituted thiosemicarbazide derivative **11a**. On the other hand, interaction of **3** with hydrazide derivatives (namely, phenyl hyrazide, isonicotinic hydrazide), the novel disubstituted thiosemicarbazide derivatives **11b,c** were obtained in good yield, respectively. Finally, the reaction of **3** with phenylsulphonylhydrazine afforded the novel disubstituted thiosemicarbazide derivative **11d** in high yield. **(**Scheme 3)

Scheme 3

3. Results and discussion

The search for newer antibacterial and antifungal agents continues due to the rapid development the resistance among bacteria and fungi to the existing antimicrobial agents. Even though novel broad spectrum antimicrobial agents were reported in recent years, the emergence of resistance has become an obstacle. Thus, more effective classes of such agents are desired. Thiourea derivatives may comprise a new class of antimicrobial agents. The aim of the present investigation is to synthesize different series of thiourea derivatives which bearing a 4-hippuric acid moiety at N^1 and various substituent at N^2. The authors measured the anti (bacterial and fungal) activities of these derivatives. They studied the effect of each substituent at N^2 on these activities and make a comparative study between them to deduce a structure activity relationship. The following selection of 23 compounds **4a-g**, **5**, **6a**,**b**, **7a-f**, **8a**,**b**, **9**, **10b** and **11b-d** were tested under the same conditions for antibacterial and antifungal activity against Gram positive and Gram negative bacteria, and fungi.

3.1. Antibacterial activities

The synthesized compounds were tested *in vitro* for antibacterial activity against the following bacterial strains: three Gram-positive bacteria, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633 and *Bacillus megaterium* ATCC 9885, and five Gram-negative bacteria, *Klebseilla peneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27953, *Escherichia coli* ATCC 25922, *Sarcina lutea, Proteous vulgaris*, and the results are

summarized in Table 1. Antimicrobial tests were carried out by the agar well diffusion method [14] using 100 μL of tested compound solution prepared by dissolving 1 mg of the chemical compound in 1 ml of dimethyl sulfoxide (DMSO). The inoculated plates were then incubated for 24 h at 37 ℃. The antibacterial agent ciprofloxacin (0.5 mg/ml) was used as a standard. After incubation time, antimicrobial activity was evaluated by measuring the inhibition zone diameters against the test organisms and compared with standard zone size ranges that determine susceptibility, intermediate susceptibility, or resistance to the screened compounds. Visual bacterial growth is observed only in areas in which the drug concentrations are below those required for growth inhibition. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

Table 1

The mean values of the inhibition zone diameter obtained for these compounds suggest that all of the thiourea derivatives evaluated possess significant antibacterial activity against most of the test organisms used in these assays. Using the general structure provided in Figure 1, certain aspects of the structure activity relationships for these compounds can be more clearly highlighted.

Figure 1

As anticipated, a clear difference in antibacterial activity is noted between compounds within and between each series, pointing to the reinforcing and opposing effects of X groups. The results of the antimicrobial screening demonstrated the following assumptions about the structural activity relationship (SAR). Structure **4**, X were alkyl and aryl moieties (**4a**; n-butyl, **4b**; cyclohexyl, **4c**; phenyl, **4d**; 3-bromophenyl, **4e**; 4-methylphenyl, **4f**; 3-fluorophenyl, **4g**; 4-fluorophenyl): Regarding the effect of X group, it is evident that varying such a unit may have a dramatic effect on antimicrobial activity, which may be augmented or reduced depending on whether a matching or mismatching relationship exists with the X group. The type of the substitutions on the benzene ring of aryl moiety is important. It was noticed that the presence of electron withdrawing groups such as fluoride and bromide at the benzene ring displayed strong effect on the antimicrobial activity. The presence of 3-bromophenyl moiety (**4d**) resulted in the highest antimicrobial activity among all the compounds investigated in this study. the presence of 3-bromophenyl moiety exhibited the highest antibacterial activity against most of the organisms, 3-bromophenyl moiety showed results greater than the reference drug reach 1.5 fold against most of the organisms. 4-Fluorophenyl moiety exhibited activities near to the references drug. Also, 3-fluorophenyl showed potent activities against *Bacillus megaterium* and good activity toward other organisms. The presence of electron donating group such as methyl at the benzene ring or leaving benzene ring without substitution had a detrimental effect on antibacterial activity. Compounds **4c** (phenyl moiety) showed moderate activity against four organisms and no activates toward the other test organisms and **4e** (4-methylphenyl moiety) showed strong activities greater than reference drug against *Sarcina lutea* only and no activates toward the

other test organisms. Also, comparing the zone inhibition diameter values obtained with **4d** (3-bromophenyl moiety) with those obtained with its **4c**; phenyl, **4e**; 4-methylphenyl, **4f**; 3-fluorophenyl, and **4g;** 4-fluorophenyl against tested organisms indicates the superiority of **4d** and much improved activity. Whereas **4c, 4e** and **4f** produced values ranging from 0-19 mm, 0-25 mm and 18-25 mm, respectively, compound **4d** afforded zone inhibition diameter values ranging from 20-32 mm. This clear difference in antibacterial activity observed between the preceding compounds is a direct reflection of the reinforcing effect induced by X group. When X was alkyl moiety, such as *n*-butyl moiety in case of **4a**, the activity was equipotent to the reference drug while cyclohexyl moiety showed strong activities against *Bacillus megaterium* and *Sarcina lutea* only and from weak to no activity towards the other test organisms

Introduction of polynuclear aromatic ring such as acridine moiety in case of compound **5** showed strong activities against all of test organisms except against *Sarcina lutea* and *Saccharomyces cervesia*.

Increasing the size of the substituent, as in structure **6**, had a detrimental effect on antibacterial activity. Structure **6**, which has benzamide side chain ending with trifluoromethylphenyl and ethoxyphenyl moieties showed good activity against two gram +ve bacteria, moderate activity against one gram -ve bacteria only. The presence of ethoxyphenyl moiety in case of **6b** showed activity more than trifluoromethyl moiety in case of **6a**.

Structure **7** has a sulfonamide moiety where X can be hydrogen; **7a,** acetyl; **7b,** $C(NH)NH_2$; **7c**, 2-thiazolyl; **7d**, 2-pyridinyl, **7e**, 2-pyrimidinyl; **7f**. The type of the alkyl or heterocyclic moieties on the sulfonamide side chain is important. The highest antibacterial activity of the sulfonamide series was observed in case of compounds **7f**. The presence of pyrimidinyl moiety showed results greater than the reference drug reaching a 1.5 fold greater zone of inhibition against most of the test organisms. The presence of thiazole nucleus resulted in the lowest antimicrobial activity among all the tested compounds; **7d** with thiazole moiety showed good activity against two gram +ve bacteria and showed result greater than the reference drug against one Gram -ve bacteria and no activates toward the other test organisms. Replacement thiazole ring by pyridine ring improve the antibacterial activity; **7e** showed strong activities near to reference drug against most of the test strains. Further introduction of polar functionality of the sulfonamide side chain, as in **7b** with acetyl moiety and **7c** with $C(NH)NH_2$ moiety, had a detrimental effect on antibacterial activity, **7b** showed strong activity against gram +ve bacteria only, **7c** showed good activities against two bacteria only. When X = was hydrogen in case of **7a** had a good effect on antibacterial activity; **7a** showed strong activities near to reference drug against most of the test strains. A similar comparison of relevant compounds from each series with the appropriate analogues clearly demonstrates the influence of X group, although the optimum effect of a synergetic combination against a particular strain may not necessarily extend to the strains.

Bis-compound **8a** with two hippuric and one thiourea moieties showed activity equipotent to the reference drug; but in case of bis-compound **8b** with two hippuric and two thiourea

moieties, the activity was increased and showed activity greater than the reference drug against *Bacillus subtilis*, *Escherichia coli*.

Compounds **9**, **10b**, the X group have substituted pyrazole moiety; introduction of pyrazole moiety resulted in increasing the antimicrobial activity. When a 4-chlorophenyldiazo moiety and amino group were attached at position 3, 4 of the heterocyclic pyrazole system caused better potency against most of the test organisms. Compound **10b** showed activity greater than reference drug against the most test organisms. The observed antimicrobial activity of compounds **10b** could be attributed to the synergistic effect of both the pyrazole and 4-chlorophenyldiazo moieties.

Structure **11**, X was NHCOPh in case **11b**, X was pyridine amide in case **11c**, X was $NHSO_2Ph$ in case **11d**. In general, treatment of **3** with hydrazide derivatives proved to be detrimental to antibacterial activity. The effect on antibacterial activity was especially apparent for the sulfonamide derivative (**11d**). Compound **11d** with sulfonamide moiety showed strong results greater than the reference drug against most of the test organisms. Compound **11b** showed good activity against most of the test organisms while **11c** showed strong activity against *Bacillus megaterium* and *Sarcina lutea* only.

The comparison between the antibacterial activities of our potent synthesized compounds and ciprofloxacin as standard antibacterial reference drug against the used Gram positive and Gram negative bacteria is represented graphically in Figure 2.

Figure 2

3.2. Antifungal activity

We chose *Saccharomyces cerevisiae* and *Candida albicans* NRRLY-477 in the antifungal susceptibility tests. Antifungal agents are evaluated against clinical isolates of standard strains of fungi by the agar well diffusion method [14] using 100 μL of tested compound solution prepared by dissolving 1 mg of the chemical compound in 1 ml of dimethyl sulfoxide. The antifungal agent ketoconazole was used as a standard at concentration 0.5 mg/mL and was tested under the same conditions. The inoculated plates were then incubated. After incubation time, antifungal activity was evaluated by measuring the inhibition zone against the test organisms and compared with that of the standard. Antifungal activities were expressed as inhibition zone diameter in millimeters (mm). The experiment was carried out in triplicate and the average zone of inhibition was calculated. The results are tabulated in Table 1. In general, some compounds were very effective antifungal agents and in several cases achieved similar or greater levels of activity as the standard antifungal agent ketoconazole (23 and 22 mm). It is noted though that some fluctuation in antibacterial activity for individual tested compound was observed across the two strains of tested fungi.

As anticipated, a clear difference in antifungal activity is noted between compounds within and between each series, pointing to the reinforcing and opposing effects of the X groups. For instance, compound **4d** (X = 3-bromophenyl**)** proved more effective against *Saccharomyces cerevisiae* (28 mm) and *Candida albicans* (30 mm) than the standard antifungal agent (23 and 22 mm). In addition, **7f, 8b, 10b** and **11d** proved more effective than ketoconazole, compound **7f** with sulfonamide side chain ending pyrimidinyl moiety produced 30 mm and 25 mm, compound **8b** gave 26 mm and 26 mm, compound **10b** with pyrazole moiety gave 25 mm and 30 mm and compound **11d** with sulfonamide moiety gave 24 mm and 32 mm inhibition zone diameter values against the two fungal strains. Thus, these compounds comprise a new class of potential broad-spectrum antifungal agents. The comparison between the antifungal activities of our potent synthesized compounds and Ketoconazole as a reference antifungal agent is illustrated graphically in Figure 3.

Figure 3

3.3. Minimum inhibitory concentrations against Gram positive bacteria, Gram negative bacteria and fungi

Minimum inhibitory concentration (MIC) of the more active synthesized compounds **4d, 4f, 7f**, **8a,b, 10b** and **11d** was then evaluated *in vitro* using the twofold serial dilution technique [15]. The lowest concentration showing no growth was taken as the minimum inhibitory concentration (MIC). The results of minimum inhibitory concentration were reported in Table 2.

Regarding the effect of X group (**4d**; 3-bromophenyl, **4f**; 3-fluorophenyl, **7f**, sulfonamide side chain ending with 2-pyrimidinyl; **8a;** hippuric acid moiety, **8b;** phenyl thiourea side chain ending with hippuric acid moiety, **10b**; pyrazole moiety; **11d;** $NHSO_2Ph$) against bacterial and fungal strains, results of antimicrobial activity in this study revealed that: The presence of 3-bromophenyl moiety (**4d**) resulted in the highest antimicrobial activity among all the compounds investigated in this study. Compound **4d** showed better results when compared with ciprofloxacin as revealed from its MIC values (3.12 - 12.5 μg/mL). Compound **4d** showed twofold potency of ciprofloxacin in inhibiting the growth of *B. subtilis* (MIC 3.12 μg/mL), *B. megaterium* (MIC 3.12 μg/mL), *K. peneumoniae* (MIC 6.25 μg/mL), *E. coli* (MIC 6.25 μg/mL) and *P. vulgaris* (MIC 6.25 μg/mL). Compound **4d** was equipotent to the ciprofloxacin in inhibiting the growth of *S. aureus*, *P. aeruginosa* and *S. lutea* (MIC 12.5 μg/mL). Regarding the activity of **4d** against fungal strains; **4d** showed twofold potency of ketoconazole in inhibiting the growth of *C. albicans* (MIC 3.12 μg/mL), and it showed 50% less potent effect than ketoconazole against *S. cerevisia* (MIC 6.25 μg/mL).

Also, compounds **7f, 10b** and **11d** showed better results when compared with ciprofloxacin as revealed from their MIC values (3.12 - 12.5 μg/mL). The presence of pyrimidinyl moiety (**7f**) showed results greater than the reference drug reaching fourfold against *P. aeruginosa* (MIC 3.12 μg/mL), and twofold against *S. aureus* (MIC 3.12 μg/mL) and *E. coli* (MIC 6.25

μg/mL). Compound **7f** was equipotent to the ciprofloxacin in inhibiting the growth of *K. peneumoniae, S. lutea* and *P. vulgaris* (MIC 12.5 μg/mL), and displayed 50% less activity compared to ciprofloxacin against the growth of the other organisms. Compound **10b** with pyrazole moiety showed twofold potency of ciprofloxacin in inhibiting the growth of *B. subtilis* (MIC 3.12 μg/mL), *E. coli* (MIC 6.25 μg/mL) and *P. vulgaris* (MIC 6.25 μg/mL), and was equipotent to the ciprofloxacin toward other organisms. Compound **11d** with sulfonamide moiety showed twofold potency of ciprofloxacin in inhibiting the growth of *B. subtilis* (MIC 3.12 μg/mL), *B. megaterium* (MIC 3.12 μg/mL) and *E. coli* (MIC 6.25 μg/mL), and was equipotent to the ciprofloxacin toward other organisms. Regarding the activity of above compounds against fungal strains; compounds **7f, 10b** and **11d** showed good results when compared with ketoconazole as revealed from their MIC values (3.12- 12.5 μg/mL). Compounds **7f** was equipotent to the ketoconazole toward *S. cerevisia* (MIC 3.12 μg/mL), and it showed 50% less potent effect than ketoconazole against *C. albicans* (MIC 12.5 μg/mL). Compounds **10b** and **11d** showed twofold potency of ketoconazole in inhibiting the growth of *C. albicans* (MIC 3.12 μg/mL), and 25% less potent effect than ketoconazole against *S. cerevisia* (MIC 12.5 μg/mL).

Compound **4f** with 3-fluorophenyl moiety was equipotent to ciprofloxacin against the growth of the different Gram-negative bacteria (MIC 12.5 μg/mL). Compound **4f** displayed 50% less activity compared to ciprofloxacin against the growth of the different Gram-positive bacteria (MIC 12.5 μg/mL) except against *S. aureus* (MIC 50 μg/mL). Regarding the activity of the **4f** against fungal strains; it showed 50% less potent effect than ketoconazole against *C. albicans* (MIC 12.5 μg/mL), and moderate potency (MIC 25 μg/mL) against *S. cerevisia* when compared with the reference drug (MIC 3.12 μg/mL).

Biscompounds **8a,b** showed potency equipotent to the ciprofloxacin toward *K. peneumoniae* and *P. aeruginosa* (MIC 12.5 μg/mL), and moderate activity against the growth of the different bacteria (MIC 12.5 - 50 μg/mL). Regarding the activity of **8a,b** against fungal strains; biscompound **8a,b** were 50% less potent effect than ketoconazole against *C. albicans* (MIC 12.5 μg/mL), and moderate potency (MIC 25 - 50 μg/mL) against *S. cerevisia* when compared with the reference drug (MIC 3.12 μg/mL).

As shown in Table 2, most of the tested compounds revealed MIC 12.5 μg/ml against *Klebseilla peneumoniae, Pseudomonas aeroginosa* and *Sarcina lutea.* The lowest MIC values were obtained against the *Candida albicans* (MIC 3.12 - 12.5 μg/mL) followed by *Bacillus megaterium, Pseudomonas aeroginosa, Klebseilla peneumoniae*. Compounds **4d, 7f, 10b** and **11d** were consistently most of the effective against all microbial strains, producing the lowest MIC (3.12 μg/ml) against most of tested organisms. In certain cases, such values are low enough to render such agents as potential candidates for further studies.

Table 2

4. Conclusion

In summary, the aim of the present investigation is to synthesize different series of thiourea derivatives which bearing a 4-hippuric acid moiety at N^1 and various substituent at N^2 through the reaction of 4-hippuric acid isothiocyanate with various nitrogen nucleophiles to achieve the antimicrobial effect and reducing the toxicity associated with thiourea. This coupling may lead to gives an opportunity in medicinal chemistry to improve the clinical and therapeutic effectiveness of a drug that is suffering from some undesirable properties hindering its clinical usefulness. The antimicrobial screening for the most of tested compounds exhibited significant antimicrobial effect. The results of the antimicrobial screening demonstrated the following assumptions about the structural activity relationship (SAR). When aryl substituent at N^2 containing electron withdrawing halogen such as fluoride and bromide displayed strong effect on the antimicrobial activity, For example, the presence of 3-bromophenyl moiety resulted in the highest antimicrobial activity among all the compounds investigated in this study. the presence of 3-bromophenyl moiety exhibited the highest antibacterial activity against most of the organisms, 3-bromophenyl moiety showed results greater than the reference drug reach 1.5 fold against most of the organisms. While, the presence of electron donating group such as methyl at the benzene ring or leaving benzene ring without substitution had a detrimental effect on antibacterial activity. When X was alkyl moiety the activity was equipotent to the reference drug. Introduction of polynuclear aromatic ring showed strong activities against all of test organisms. Increase in the size of X group had a detrimental effect on antibacterial activity. Coupling of thiourea with sulfonamide showed strong activities near to reference drug against most of the test strains. Biscompound with two hippuric and one thiourea moieties showed activity equipotent to the reference drug. Introduction of pyrazole moiety resulted in increasing the antimicrobial activity. In general, treatment of isothiocyanate derivative with hydrazide derivatives proved to be detrimental to antibacterial activity. The effect on antimicrobial activity was especially apparent for the sulfonamide derivative. Coupling with sulfonamide moiety showed strong results greater than the reference drug against most of the test organisms.

5. Experimental Section

All melting points were determined on an electrothermal Gallenkamp apparatus and are uncorrected. The IR spectra were measured on a Mattson 5000 FTIR Spectrometer in potassium bromide discs. The NMR spectra were recorded in DMSO-d_6 on a Bruker WP spectrometer (500 MHz) and the chemical shifts δ downfield from TMS as an internal standard. The mass spectra were recorded on Finnegan MAT 212 instrument, the ionizing voltage was 70 ev, at Faculty of Science, Cairo University. Elemental analyses were carried out by the Microanalytical unit of Faculty of Science, Cairo University.

*5.1 Synthesis of 2-(4-thioureidobenzamido)acetic acid (**2**)*

To a magnetically stirred solution of 4-aminohippuric acid **1** (0.01 mol) in 10 mL of 6 N HCl was added ammonium thiocyanate (1.14 g, 0.015 mol). The mixture was heated at 80 °C under reflux for 12 h until abundant precipitate appeared. The mixture was cooled to room

temperature, the solid product obtained was collected by filtration, washed with water, and dried under vacuum to produce the corresponding thiourea **2** as colorless crystals; yield 55%; mp 135-136 °C; IR: ν/cm^{-1}: 3412, 3270, 3160 (NH, NH_2, OH), 1721, 1645 (C=O); ^{1}HNMR: δ/ppm: 3.90 (d, 2H, *J* = 6.22 Hz, CH_2), 5.40 (br, 2H, NH_2), 7.10 - 7.70 (m, 4H, Ar-H), 8.83 (t, 1H, *J* = 6.13 Hz, CONH), 10.84 (s, 1H, NH), 12.22 (br, 1H, OH); ^{13}C NMR: 40.2 ($NHCH_2$), 116.8, 117.8, 126.6 (2C), 130.2, 141.9, 166.5 (CONH), 171.9 (COOH), 176.6 (C=S); MS, m/z (%): 253 (M^+, 12.8), 192 (10.4), 160 (23.4), 128 (33.1), 96 (19.86), 64 (100); Anal. calcd for $C_{10}H_{11}N_3O_3S$ (253.05): C, 47.42; H, 4.38; N, 16.59. Found: C, 47.50; H, 4.40; N, 16.70%.

*5.2 Synthesis 2-(4-isothiocyanatobenzamido)acetic acid (**3**)*

To an efficiently stirred solution of (4-amino-benzoylamino)-acetic acid **1** (0.12 mol) in 150 ml H_2O and 50 ml conc. HCl, thiophosegene (0.12 mol) was added in one portion and the stirring was continued until the red color of thiophosegene disappeared and white crystals of isothiocyanate began to precipitate (0.5-1.0 h). The solid product obtained was collected by filtration and crystallized from THF to give the corresponding isothiocyanato derivative **3** as colorless crystals; yield 74%; mp 194-196 °C; IR: ν/cm^{-1}: 3454 (OH), 3259 (NH), 1733, 1638 (C=O); ^{1}H NMR: δ/ppm: 4.00 (d, 2H, *J* = 6.10 Hz, CH_2), 7.58 (d, 2H, *J* = 8.45 Hz, Ar-H), 8.14 (d, 2H, *J* = 8.40 Hz, Ar-H), 9.04 (t, 1H, *J* = 6.15 Hz, CONH), 12.66 (br, 1H, OH); MS, m/z (%): 236 (M^+, 8.4), 192 (M-CO_2; 24.4), 163 (M - NCH_2COOH; 11.1), 162 (M - $HNCH_2COOH$; 100), 134 (C_6H_4NCS; 38.4); Anal. Calcd for $C_{10}H_8N_2O_3S$ (236.03): C, 50.84; H, 3.41; N, 11.86. Found: C, 51.00; H, 3.50; N, 11.70%.

*5.3 Synthesis of disubstitued thiourea derivatives **4a-g***

Method A: a solution of isothiocyanate **3** (0.01 mol) in dioxane was treated with the requisite amine (0.01 mol) (namely *n*-butyl amine, cyclohexyl amine, aniline, 3-bromoaniline, 4-methylaniline, 3-fluoroaniline, 4-fluoroaniline). The reaction mixture was heated under reflux for 3 hours. The solution was concentrated under vacuum. The solid product so formed after cooling was filtered off, washed with ethanol, dried well, and recrystallized from dioxane to give **4a-g** in good yield as shown below.

Method B: a solution of amine **1** (0.01 mol) and the requisite isothiocyanate (namely *n*-butyl isothiocyanate, cyclohexyl isothiocyanate, phenyl isothiocyanate, 3-bromophenyl isothiocyanate, 4-methylphenyl isothiocyanate, 3-fluorophenyl isothiocyanate, 4-fluorophenyl isothiocyanate) (0.01 mol) in dioxane was heated under reflux for 3 hours. The solution was concentrated under vacuum, left to cool. The solid product was filtered off, washed with ethanol, dried well, and recrystallized (m.p. and mixed m.p.).

*5.3.1 2-(4-(3-Butylthioureido)benzamido)acetic acid (**4a**)* as colorless crystals; yield 74%; mp 169-171 °C; IR: ν/cm^{-1} = 3443, 3318, 3173 (NH, OH), 1709, 1641 (C=O); ^{1}H NMR: δ/ppm: 0.93 (t, 3H, butyl-H at CH_3), 1.30-1.60 (m, 4H, butyl-H at $C_{2,3}$-H), 3.58 (m, 2H, butyl-H at C_1-H), 3.85 (d, 2H, *J* = 6.40 Hz, CH_2), 7.54 (d, 2H, *J* = 8.40 Hz, Ar-H), 7.69 (d, 2H, *J* =

8.40 Hz, Ar-H), 8.72 (t, 1H, *J* = 6.40 Hz, CONH), 9.35 (s, 1H, NH), 9.90 (s, 1H, NH), 12.30 (br, 1H, OH); ^{13}C NMR: 14.18 (butyl CH_3), 20.05 (butyl C_3), 32.30 (butyl C_2), 39.83 ($NHCH_2$), 44.17 (butyl C_1), 113.1 (2C), 117.0, 128.7, 129.3 (2C), 152.3 (CONH), 166.7 (COOH), 172.4 (C=S); Anal. calcd for $C_{14}H_{19}N_3O_3S$ (309.38): C, 54.35; H, 6.19; N, 13.58. Found: C, 54.20; H, 6.00; N, 13.40 %.

*5.3.2 2-(4-(3-Cyclohexylthioureido)benzamido)acetic acid (**4b**)* as colorless crystals; yield 76%; mp 180-181°C; IR: ν/cm^{-1} = 3478, 3235, 3192 (NH, OH), 1731, 1645 (C=O); ^{1}H NMR: δ/ppm: 1.10 - 1.95 (m, 10H, cycl-H), 3.93 (d, 2H, *J* = 5.49 Hz, CH_2), 4.20 (m, 1H, cycl-C_1-H), 7.55 (d, 2H, *J*= 8.40 Hz, Ar-H), 7.77 (d, 2H, *J*= 8.40 Hz, Ar-H), 8.72 (t, 1H, *J*= 5.40 Hz, CONH), 8.87 (s, 1H, NH), 9.90 (s, 1H, NH), 12.30 (br, 1H, OH); ^{13}C NMR: 25.36 (2C), 25.57, 32.2 (2C), 41.23, 52.6, 113.1 (2C), 121.1, 129.3 (2C), 143.2, 166.5 (CONH), 172.0 (COOH), 179.4 (C=S); Anal. calcd for $C_{16}H_{21}N_3O_3S$ (335.42): C, 57.29; H, 6.31; N, 12.53. Found: C, 57.30; H, 6.20; N, 12.40 %.

*5.3.3 2-(4-(3-Phenylthioureido)benzamido)acetic acid (**4c**):* as colorless crystals; yield 76%; mp 220-224 °C; IR: ν/cm^{-1} = 3425-3173 (NH, OH), 1708, 1645 (C=O); ^{1}HNMR: δ/ppm: 3.87 (d, 2H, *J* = 6.40 Hz, CH_2), 7.15 - 7.83 (m, 9H, Ar-H), 8.97 (t, 1H, *J* = 6.45 Hz, CONH), 9.77 (br, 1H, NH), 9.93 (br, 1H, NH), 12.12 (br, 1H, OH); Anal. calcd for $C_{16}H_{15}N_3O_3S$ (329.08): C, 58.34; H, 4.59; N, 12.76. Found: C, 56.37; H, 3.74; N, 18.28 %.

*5.3.4 2-(4-(3-(3-Bromophenyl)thioureido)benzamido)acetic acid (**4d**):* as colorless crystals; yield 87%; mp > 300 °C; IR: ν/cm^{-1} = 3451, 3140 (NH, OH), 1711, 1645 (C=O); ^{1}HNMR: δ/ppm: 3.83 (d, 2H, *J* = 6.31 Hz, CH_2), 6.85 - 7.88 (m, 8H, Ar-H), 8.77 (t, 1H, *J* = 6.39 Hz, CONH), 9.85 (s, 1H, NH), 10.42 (s, 1H, NH), 12.22 (br, 1H, OH); Anal. calcd for $C_{16}H_{14}BrN_3O_3S$ (406.99): C, 47.07; H, 3.46; N, 10.29. Found: C, 47.00; H, 3.50; N, 10.20 %.

*5.3.5 2-(4-(3-p-Tolylthioureido)benzamido)acetic acid (**4e**):* as colorless crystals; yield 91%; mp 205-207 °C; IR: ν/cm^{-1} = 3492, 3225, 3191 (NH, OH), 1703, 1645 (C=O); ^{1}HNMR: δ/ppm: 2.24 (s, 3H, CH_3), 3.87 (d, 2H, *J* = 6.45 Hz, CH_2), 7.10 (d, 2H, *J* = 8.40 Hz, Ar-H), 7.30 (d, 2H, *J* = 6.20 Hz, Ar-H), 7.58 (d, 2H, *J* = 8.40 Hz, Ar-H), 7.79 (d, 2H, *J* = 8.40 Hz, Ar-H), 8.73 (t, 1H, *J* = 6.45 Hz, CONH), 9.85 (s, 1H, NH), 9.90 (s, 1H, NH), 12.32 (br, 1H, OH); Anal. calcd for $C_{17}H_{17}N_3O_3S$ (343.10): C, 59.46; H, 4.99; N, 12.24. Found: C, 59.30; H, 4.10; N, 12.20 %.

*5.3.6 2-(4-(3-(3-Fluorophenyl)thioureido)benzamido)acetic acid (**4f**):* as colorless crystals; yield 87%; mp > 300 °C; IR: ν/cm^{-1} = 3440, 3212, 3173 (NH, OH), 1721, 1645 (C=O); ^{1}HNMR: δ/ppm: 3.90 (d, 2H, *J* = 6.40 Hz, CH_2), 6.95-7.91 (m, 8H, Ar-H), 8.87 (t, 1H, *J* = 6.40 Hz, CONH), 10.95 (s, 1H, NH), 11.18 (s, 1H, NH), 12.32 (br, 1H, OH); ^{13}C NMR: 41.14 ($NHCH_2$), 115.1, 116.3, 121.0, 122.4 (2C), 127.3, 128.5 (2C), 129.9, 137.3, 143.4, 160.7 (C-F), 166.5 (CONH), 171.9 (COOH) 180.3(C=S); Anal. calcd for $C_{16}H_{14}FN_3O_3S$ (347.07): C, 55.32; H, 4.06; N, 12.10. Found: C, 55.30; H, 4.10; N, 12.00 %.

*5.3.7 2-(4-(3-(4-Fluorophenyl)thioureido)benzamido)acetic acid (**4g**):* as colorless crystals; yield 80%; mp > 300 °C; IR: ν/cm^{-1} = 3343 - 3198 (NH, OH), 1724, 1640 (C=O); ^{1}HNMR: δ/ppm: 3.90 (d, 2H, *J* = 6.45 Hz, CH_2), 6.91-7.89 (m, 8H, Ar-H), 8.85 (t, 1H, *J* = 6.45 Hz CONH), 10.92 (s, 1H, NH), 11.42 (s, 1H, NH), 12.32 (br, 1H, OH); ^{13}C NMR: 41.23 ($NHCH_2$), 115.5, 115.7, 122.8 (2C), 126.8 (2C), 128.2 (2C), 129.7, 136.0, 142.8, 160.7 (C-F), 166.5 (CONH), 171.9 (COOH) 180.3 (C=S); Anal. calcd for $C_{16}H_{14}FN_3O_3S$ (347.07): C, 55.32; H, 4.06; N, 12.10. Found: C, 55.40; H, 4.00; N, 12.00 %.

*5.4 Synthesis of disubstitued thiourea derivatives **5** and **6a,b***

A solution of isothiocyanate **3** (0.01 mol) in 30 ml dioxane, 9-aminoacridine, 2-amino-*N*-(3-trifluoromethylphenyl)-benzamide or 2-amino-*N*-(4-ethoxyphenyl)-benzamide (0.01 mol) was added. The reaction mixture was heated under reflux for 3 hours. The solution was concentrated under vacuum, left to cool. The solid product so formed was filtered off, washed with ethanol, dried well, and recrystallized from ethanol/dioxane.

*5.4.1 Synthesis of 2-(4-(3-acridin-9-ylthioureido)benzamido)acetic acid (**5**):* as yellow crystals, yield 85%; mp > 300 °C; IR: ν/cm^{-1} = 3415, 3218, 3187 (NH, OH), 1731, 1645 (C=O); ^{1}HNMR: δ/ppm: 4.04 (d, 2H, *J* = 5.74 Hz, CH_2), 6.98 - 8.94 (m, 13H, 12Ar-H + CONH), 11.02 (s, 1H, NH), 11.51 (s, 1H, NH), 12.12 (br, 1H, OH); Anal. calcd for $C_{23}H_{18}N_4O_3S$ (430.11): C, 64.17; H, 4.21; N, 13.01. Found: C, 64.30; H, 4.50; N, 13.20 %.

*5.4.2 2-(4-(3-(2-(3-(Trifluoromethyl)phenylcarbamoyl)phenyl)thioureido)benzamido)acetic acid (**6a**):* as colorless crystals; yield 87%; mp 265-270 °C; IR: ν/cm^{-1} = 3412- 3187 (NH, OH), 1722, 1645 (C=O); ^{1}H NMR: δ/ppm: 3.87 (d, 2H, *J* = 5.80 Hz, CH_2), 6.40 - 8.88 (m, 13H, 12Ar-H + CONH), 9.92 (s, 1H, NH), 9.95 (s, 1H, NH), 10.62 (br, 1H, NH), 12.41 (br, 1H, OH); ^{13}C NMR: 41.3 ($NHCH_2$), 116.3 (2C),, 116.7, 124.9 (2C), 127.9, 128.5 (4C), 129.7 (4C), 134.2, 136.2 (2C), 140.1, 142.4, 160.2 (CONH), 166.6 (CONH), 171.9 (COOH), 176.2 (C=S); MS, m/z (%): 516 (M^+, 84), 490 (89.0), 463 (94.0), 289 (88.1), 219 (99), 202 (99), 71 (51); Anal. calcd for $C_{24}H_{19}F_3N_4O_4S$ (516.11): C, 55.81; H, 3.71; N, 10.85. Found: C, 55.70; H, 3.60; N, 10.60 %.

*5.4.3 2-(4-(3-(2-(4-Ethoxyphenylcarbamoyl)phenyl)thioureido)benzamido)acetic acid (**6b**):* as colorless crystals; yield 86%; mp >300 °C; IR: ν/cm^{-1} = 3418, 3300, 3150 (NH,OH), 1715, 1642 (2C=O); ^{1}H NMR: δ/ppm: 1.25 (t, 3H, *J* = 6.85 Hz, $CH_2\underline{CH_3}$), 3.80 - 4.04 (m, 4H, $2CH_2$), 6.40 - 8.82 (m, 13H, 12Ar-H + CONH), 9.88 (s, 1H, NH), 9.98 (s, 1H, NH), 10.76 (br, 1H, NH), 12.52 (br, 1H, OH); Anal. calcd for $C_{25}H_{24}N_4O_5S$ (492.15): C, 60.96; H, 4.91; N, 11.37. Found: C, 61.10; H, 4.70; N, 11.60 %.

*5.5 Synthesis of sulfonamido thiourea derivatives **7a-f***

A mixture of **3** (0.01 mol), the appropriate sulfa drugs (namely sulfanilamide, sulfacetanilide, sulfaguanidine, sulfathiazole, sulfapyridine and sulfadiazine) in dioxane (30 mL) was heated

under reflux for 3 hours. The solution was concentrated under vacuum, left to cool. The resulting solid was filtered off and recrystallized from diaxone.

*5.5.1 2-(4-(3-(4-Sulfamoylphenyl)thioureido)benzamido)acetic (**7a**):* as beige crystals; yield 79%; mp 205-207 °C; IR: ν/cm^{-1} = 3450, 3300, 3200 (NH, NH_2, OH), 1710, 1642 (C=O); ^{1}HNMR: δ/ppm: 3.95 (d, 2H, *J* = 5.94 Hz, CH_2), 6.94 - 8.04 (m, 10H, 8Ar-H + SO_2NH_2), 8.82 (t, 1H, *J* = 6.11 Hz, CONH), 10.88 (s, 1H, NH), 11.32 (s, 1H, NH), 12.22 (br, 1H, OH); ^{13}C NMR: 41.3 ($NHCH_2$), 122.9 (2C), 123.3 (2C), 126.7 (2C), 128.2 (2C), 130.0, 139.8, 142.6, 143.0, 166.6 (CONH), 171.9 (COOH) 180.0(C=S); MS, m/z (%): 408 (M^+, 7.0), 401 (11.0), 268 (12.1), 214 (34.7), 88 (100); Anal. calcd for $C_{16}H_{16}N_4O_5S_2$ (408.06): C, 47.05; H, 3.95; N, 13.72. Found: C, 47.10; H, 4.10; N, 13.60 %.

*5.5.2 2-(4-(3-(4-(N-Acetylsulfamoyl)phenyl)thioureido)benzamido)acetic acid (**7b**)***:** as beige crystals; yield 67%; mp >300 °C; IR: ν/cm^{-1} = 3423-3110 (NH, OH), 1714, 1641 (C=O); ^{1}HNMR: δ/ppm: 1.87 (s, 3H, $COCH_3$), 4.03 (d, 2H, *J* = 6.24 Hz, CH_2), 7.50 - 8.74 (m, 9H, 8Ar-H + NH), 9.08, 10.32, 11.73, 12.43 (br, 4H, 3NH, OH); MS, m/z (%): 450 (M^+, 62.0), 419 (94.0), 325 (92.1), 275 (100), 248 (95), 188 (94), 55 (98); Anal. calcd for $C_{18}H_{18}N_4O_6S_2$ (450.07): C, 47.99; H, 4.03; N, 12.44. Found: C, 47.80; H, 4.10; N, 12.60 %.

*5.5.3 2-(4-(3-(4-(N-Carbamimidoylsulfamoyl)phenyl)thioureido)benzamido)acetic acid (**7c**):* as beige crystals; yield 66%; mp >300 °C; IR: ν/cm^{-1} = 3400 - 3160 (NH, NH_2, OH), 1763, 1645 (C=O); ^{1}HNMR: δ/ppm: 3.87 (s, 1H, NH), 4.03 (m, 2H, CH_2), 6.66 (br, 2H, NH_2), 7.53 - 8.74 (m, 9H, 8Ar-H + CONH), 9.17 (s, 1H, NH), 10.33 (m, 2H, 2NH), 12.43 (br, 1H, OH); MS, m/z (%): 450 (M^+, 57.34), 419 (66.4), 399 (60.8), 306 (60.1), 261 (64.4), 194 (65.7), 80 (93.6), 64 (100); Anal. calcd for $C_{17}H_{18}N_6O_5S_2$ (450.08): C, 45.32; H, 4.03; N, 18.66. Found: C, 45.50; H, 4.20; N, 18.60 %.

*5.5.4 2-(4-(3-(4-(N-Thiazol-2-ylsulfamoyl)phenyl)thioureido)benzamido)acetic acid (**7d**)***:** as beige crystals; yield 70%; mp > 300 °C; IR: ν/cm^{-1} = 3450, 3254, 3150 (NH, OH), 1733, 1645 (C=O); ^{1}H NMR: δ/ppm: 3.88 (d, 2H, *J* = 5.89 Hz, CH_2), 6.98 - 8.79 (m, 12H, 10Ar-H + 2NH), 10.79 (s, 1H, NH), 11.14 (s, 1H, NH), 12.08 (br, 1H, OH); Anal. calcd for $C_{19}H_{17}N_5O_5S_3$ (491.04): C, 46.42; H, 3.49; N, 14.25. Found: C, 46.60; H, 3.60; N, 14.50 %.

*5.5.5 2-(4-(3-(4-(N-Pyridin-2-ylsulfamoyl)phenyl)thioureido)benzamido)acetic acid (**7e**):* as beige crystals; yield 74%; mp > 300 °C; IR: ν/cm^{-1} = 3386, 3250 (NH, OH), 1721, 1645 (C=O); ^{1}H NMR: δ/ppm: 3.88 (d, 2H, *J* = 6.10 Hz, CH_2), 7.03 - 8.86 (m, 14H, 12Ar-H + 2NH), 10.79 (s, 1H, NH), 11.14 (s, 1H, NH), 12.08 (br, 1H, OH); Anal. calcd for $C_{21}H_{19}N_5O_5S_2$ (485.08): C, 51.95; H, 3.94; N, 14.42. Found: C, 52.10; H, 3.70; N, 14.50 %.

*5.5.6 2-(4-(3-(4-(N-Pyrimidin-2-ylsulfamoyl)phenyl)thioureido)benzamido)acetic acid (**7f**):* as beige crystals; yield 76%; mp 255-258 °C; IR: ν/cm^{-1} = 3325, 3200, 3160, (NH, OH), 1705 (C=O), 1691, 1645 (C=O); ^{1}H NMR: δ/ppm: 3.88 (d, 2H, *J* = 5.79 Hz, CH_2), 6.73 - 8.86 (m, 13H, 12Ar-H + 2NH), 11.64 (s, 2H, 2NH), 12.08 (br, 1H, OH); Anal. calcd for $C_{20}H_{18}N_6O_5S_2$ (486.52): C, 49.37; H, 3.73; N, 17.27. Found: C, 49.50; H, 3.60; N, 17.10.

5.6 Synthesis of bis-hippuric acid ***8a,b***

A mixture of isothiocyanate **3** (0.02 mol) and 4-aminohippuric acid **1** (0.02 mol) or 1,4-phenylenediamine (0.01 mol) in dioxane (30 ml) was heated under reflux for 3 hours, then allowed to cool. The solid product so formed was filtered off, washed with ethanol, dried well, and recrystallized from dioxane to give the bis-compound **8a,b,** respectively**.**

*5.6.1 Synthesis of N,N'-bis(4-hippuric acid)thiourea (**8a**):* colorless crystals; yield 75%; mp 260-261 °C; IR: ν/cm^{-1}= 3243, 3198, 3161 (NH, OH), 1694, 1638 (C=O); ^{1}HNMR: δ/ppm: 3.53 (m, 4H, 2CH_2), 6.68 - 8.58 (m, 10H, 8Ar-H + 2NH), 11.27 (br, 2H, 2NH), 12.32 (br, 2H, 2OH); Anal. Calcd for $C_{19}H_{18}N_4O_6S$ (430.09): C, 53.02; H, 4.22; N, 13.02. Found: C, 53.00; H, 4.00; N, 13.00%.

*5.6.2 Synthesis of 3,3'-bis(4-hippuric acid) 1,1'-(1,4-phenylene)dithiourea (**8b**):* pale brown crystals; yield 71%; mp 270-272 °C; IR: ν/cm^{-1} = 3450-3173 (NH, OH), 1712, 1641 (C=O); ^{1}HNMR: δ/ppm: 3.53 (m, 4H, 2CH_2), 6.68 - 8.58 (m, 14H, 12Ar-H + 2CONH), 11.27 (m, 4H, 4NH), 12.32 (br, 2H, 2OH); MS, m/z (%): 580 (M^+, 50.8), 575 (100), 501 (87.7), 301 (85.1), 102 (99.1), 61 (95.1); Anal. Calcd for $C_{26}H_{24}N_6O_6S_2$ (580.12): C, 53.78; H, 4.17; N, 14.47. Found: C, 53.70; H, 4.00; N, 14.40%.

5.7 Synthesis of pyrazole derivatives ***9, 10a,b***

A mixture of isothiocyanate **3** (0.01 mol) and aminopyrazole derivatives (namely 4-aminoantipyrene, 5-amino-3-phenylamino-1*H*-pyrazole-4-carboxylic acid amide and 4-(4-chloro-phenylazo)-4*H*-pyrazole-3,5-diamine) (0.01mol) in dioxane (30 mL) was heated under reflux for 3h. The reaction mixture was concentrated under vacuum, left to cool and then cold ethanol was added. The solid product obtained was filtered off and crystallized from ethanol.

*5.7.1 2-(4-(3-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)thioureido)benzamido)acetic acid (**9**):* as colorless crystals; yield 73%; mp 240-241°C; IR: ν/cm^{-1}: 3360, 3265, 3146 (NH, OH), 1697, 1665 (C=O); ^{1}H NMR: δ/ppm: 2.05 (s, 3H, CH_3), 3.08 (s, 3H, CH_3), 3.87 (d, 2H, *J* = 5.35 Hz, CH_2), 7.29 - 7.84 (m, 9H, Ar-H), 8.74 (t, 1H, *J* = 5.40 Hz, CONH), 9.25 (br, 1H, NH), 9.85 (br, 1H, NH), 12.38 (br, 1H, OH); MS, m/z (%): 439 (M^+, 9.4), 162 (54.7), 120 ($C_6H_4CONH_2$; 100), 56 (67.8); Anal. Calcd for $C_{21}H_{21}N_5O_4S$ (439.13): C, 57.39; H, 4.82; N, 15.94. Found: C, 57.40; H, 4.90; N, 16.00%.

*5.7.2 2-(4-(3-(4-carbamoyl-3-(phenylamino)-1H-pyrazol-5-yl)thioureido)benzamido)acetic acid(**10a**):* as yellow crystals; yield 71%; mp 245-247 °C; IR: ν/cm^{-1}: 3432 - 3110 (NH, NH_2, OH), 1709, 1640 (C=O); ^{1}HNMR: δ/ppm: 3.88 (d, 2H, *J* = 6.10 Hz, CH_2), 5.81 (br, 2H, NH_2), 6.69 - 7.45 (m, 9H, 7Ar-H + 2NH), 7.81 (d, 2H, *J* = 7.65 Hz, Ar-H), 8.77 (t, 1H, *J* = 6.10 Hz, CONH), 8.98 (s, 1H, NH), 11.27 (s, 1H, NH), 11.82 (br, 1H, OH); Anal. Calcd for $C_{20}H_{19}N_7O_4S$ (453.12): C, 52.97; H, 4.22; N, 21.62. Found: C, 52.90; H, 4.20; N, 21.50%.

*5.7.3 2-(4-(3-(3-amino-4-((4-chlorophenyl)diazenyl)-1H-pyrazol-5-yl)thioureido)benzamido)acetic acid (**10b**):* as yellow crystals; yield 76%; mp 201-203 °C; IR: ν/cm^{-1}: 3400-3160 (NH, NH_2, OH), 1711, 1639 (C=O); ^{1}H NMR: δ/ppm: 3.88 (d, 2H, *J* = 6.10 Hz, CH_2), 6.32 (br, 2H, NH_2), 7.36 - 7.77 (m, 8H, Ar-H), 8.76 (t, 1H, *J* = 6.15 Hz, CONH), 10.67 (br, 1H, NH), 11.26 (br, 1H, NH), 11.87 (br, 1H, NH), 11.92 (br, 1H, OH); MS, m/z (%): 472 (M^+, 5.7), 301 (5.8), 297 (4.1), 236 (6.6), 205 (6.7), 162 (18.1), 95 (23.1), 69 (100); Anal. calcd for $C_{19}H_{17}ClN_8O_3S$ (472.08): C, 48.26; H, 3.62; N, 23.69. Found: C, 48.20; H, 3.60; N, 23.50%.

*5.8 Synthesis of disubstituted thiosemicarbazide derivatives **11a-d***

A mixture of isothiocyanate **3** (0.01 mol) and hydrazine derivatives (0.01 mol) (namely phenylhydrazine, phenylhydrazide, isonicotinohydrazide and phenylsulfonylhydrazine) (0.01 mol) in ethanol (30 mL) was heated under reflux for one hour. The solid product was filtered of, washed with ethanol, dried well, and recrystallized from ethanol/dioxane

*5.8.1 2-(4-(2-Phenylhydrazinecarbothioamido)benzamido)acetic acid (**11a**):* a**s** colorless crystals, yield (88%); mp 165-166 ^{o}C**;** IR: ν/cm^{-1} = 3441 - 3161 (NH, OH), 1700, 1641 (C=O);); ^{1}HNMR: δ/ppm: 3.93 (d, 2H, *J* = 5.88 Hz, CH_2), 6.65 - 8.75 (m, 11H, 9Ar-H + 2NH), 9.87 (br, 1H, NH), 10.72 (br, 1H, NH), 12.04 (br, 1H, OH); MS, m/z (%): 344 (M^+, 14.7), 236 (11.4), 192 (27.4), 162 (100), 108 (92.5), 65 (85.6); Anal. Calcd for $C_{16}H_{16}N_4O_3S$ (344.09): C, 55.80; H, 4.68; N, 16.27. Found: C, 55.90; H, 4.50; N, 16.20%.

*5.8.2 2-(4-(2-Benzoylhydrazinecarbothioamido)benzamido)acetic acid (**11b**):* yellow crystals; yield (78%); mp 216-218 ^{o}C**;** IR: ν/cm^{-1} = 3440 - 3173 (NH, OH), 1698, 1638 (C=O); ^{1}HNMR: δ/ppm: 3.87 (d, 2H, *J* = 4.95 Hz, CH_2), 7.55 (d, 2H, *J* = 8.45 Hz, Ar-H), 7.80 (m, 5H, Ar-H), 8.75 (m, 3H, 2Ar-H and NH), 9.95 (br, 2H, NH), 10.88 (br, 1H, NH), 12.59 (br, 1H, OH); Anal. Calcd for $C_{17}H_{16}N_4O_4S$ (372.09): C, 54.83; H, 4.33; N, 15.04. Found: C, 54.90; H, 4.50; N, 15.20%.

*5.8.3 2-(4-(2-Isonicotinylhydrazinecarbothioamido)benzamido)acetic acid (**11c**):* colorless crystals, yield (73%); m.p. 233-235 ^{o}C**.** IR: ν/cm^{-1} = 3462 - 3191 (NH, OH), 1703, 1664 (C=O); ^{1}HNMR: δ/ppm: 3.87 (d, 2H, *J* = 4.95 Hz, CH_2), 7.55- 8.75 (m, 10H, 8Ar-H and 2NH), 9.95 (s, H, NH), 10.88 (s, 1H, NH), 12.59 (br, 1H, OH); Anal. calcd for $C_{16}H_{15}N_5O_4S$ (373.08): C, 51.47; H, 4.05; N, 18.76. Found: C, 51.60; H, 4.20; N, 18.90%.

*5.8.4 2-(4-(2-(Phenylsulfonyl)hydrazinecarbothioamido)benzamido)acetic acid (**11d**):* as colorless crystals; yield 71%; mp 161-163 °C; IR: ν/cm^{-1} = 3386-3150 (NH, OH), 1701, 1641 (C=O);); ^{1}HNMR: δ/ppm: 3.87 (d, 2H, *J* = 5.95 Hz, CH_2), 6.94 - 8.89 (m, 11H, 9Ar-H and 2NH), 10.24 (s, 1H, 1NH), 11.34 (s, 1H, NH), 12.00 (br, 1H, OH); ^{13}C NMR: 40.1 ($NHCH_2$), 124.9 (2C), 127.7 (2C), 128.5 (2C), 129.7 (2C), 130.6, 134.0, 138.2, 142.2, 166.6, 171.9, 181.3; MS, m/z (%): 408 (M^+, 23.8), 221 (40.4), 203 (39.3), 108 (100), 75 (37.1); Anal.

Calcd for $C_{16}H_{16}N_4O_5S_2$ (408.06): C, 47.05; H, 3.95; N, 13.72. Found: C, 47.20; H, 3.80; N, 13.90%.

5.9. Antimicrobial activity

Chemical compounds were individually tested against a panel of gram positive and gram negative bacterial pathogens and fungi. Antimicrobial tests were carried out by the agar well diffusion method using 100 µL of suspension containing 1 x10^8 CFU/mL of pathological tested bacteria and 1 x10^6 CFU/ml of yeast spread on nutrient agar (NA) and Sabouraud dextrose agar (SDA) media, respectively. After the media had cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 100 µL of tested compound solution prepared by dissolving 1 mg of the chemical compound in one ml of dimethyl sulfoxide (DMSO). The inoculated plates were then incubated for 24 h at 37 °C. Negative controls were prepared using DMSO employed for dissolving the tested compound. Ciprofloxacin (0.5 mg/ml) and Ketoconazole (0.5 mg/ml) were used as standard for antibacterial and antifungal activity, respectively. After incubation time, antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms and compared with that of the standard. The observed zone of inhibition is presented in Table 1. Antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm). The experiment was carried out in triplicate and the average zone of inhibition was calculated.

5.10. Minimal inhibitory concentration (MIC) measurement

Screening was performed following the procedure outlined in the Manual of Clinical Microbiology [15]. All the bacteria were incubated and activated at 30 °C for 24 h inoculation into nutrient broth and the fungi were incubated in malt extract broth for 48 h. The compounds were dissolved in DMSO and then diluted using cautiously adjusted Mueller-Hinton broth. Two-fold serial concentrations of the compounds were employed to determine the (MIC). The final concentrations of the solutions were 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/ml. In each case triplicate tests were performed and the average was taken as the final reading. The tubes were then inoculated with the test organisms, grown in their suitable broth at 37 °C for 24 hours for tested microorganisms (1×10^8 CFU/ml for bacteria and 1 x10^6 CFU/ml of fungi), each 5 ml received 0.1 ml of the above inoculum and incubated at 37 °C for 24 hours. The lowest concentration showing no growth was taken as the minimum inhibitory concentration (MIC).

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- Synthesis of *N*-(4-hippuric acid) isothiocyanate
- Using the isothiocyanate for synthesizing variously substituted thioureas.
- The antimicrobial activity assay was determined.

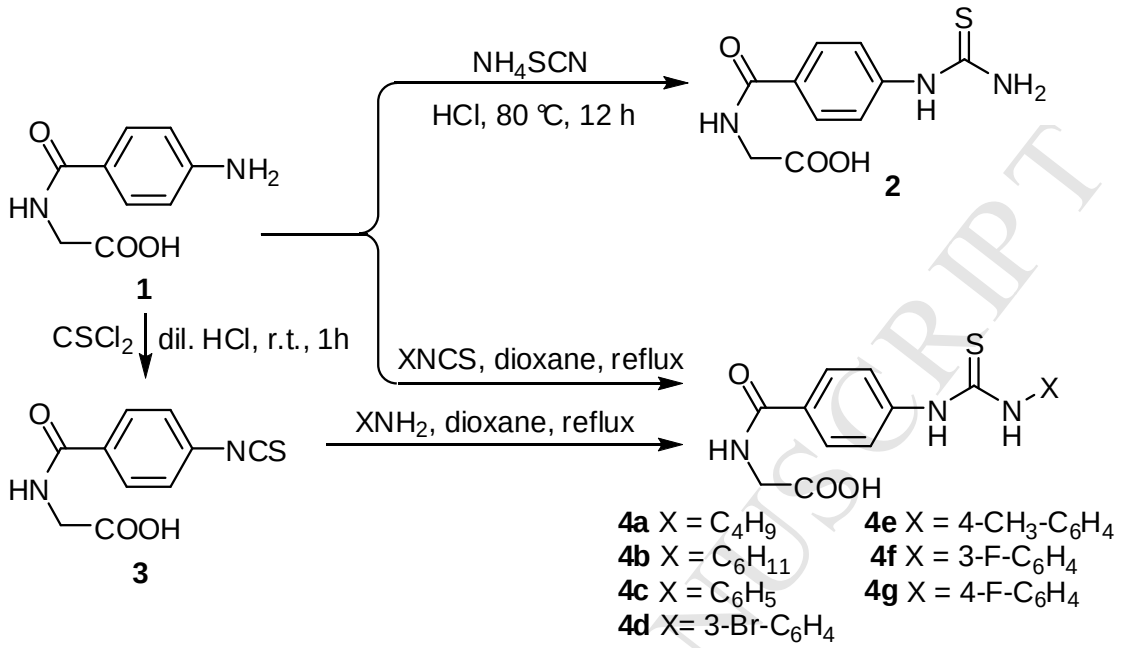
Table 1: Antimicrobial activity of the synthesized compounds against the pathological organisms expressed as inhibition diameter zones in millimeters (mm) based on well diffusion assay

Compd.	Gram +ve			Gram -ve					Fungi	
No.	*S. aureus*	*B. subtilis*	*B. megaterium*	*K. peneumoniae*	*P. aeroginosa*	*E. coli*	*S. lutea*	*P. vulgaris*	*S. cervesia*	*C. albicans*
4a	22	13	16	20	22	22	9	21	18	10
4b	0	9	23	12	0	11	24	0	9	0
4c	0	17	13	0	12	19	0	0	0	0
4d	31	30	32	30	22	27	20	24	28	30
4e	0	0	19	0	0	9	25	20	0	18
4f	20	18	27	23	21	20	20	25	17	22
4g	20	0	15	20	22	22	20	20	13	9
5	20	12	18	23	15	15	0	20	0	11
6a	0	16	14	0	0	0	0	10	15	0
6b	20	0	0	20	0	0	0	15	18	9
7a	14	18	20	22	19	18	20	20	17	0
7b	15	18	20	0	12	0	0	0	0	0
7c	0	16	20	0	0	0	0	0	15	0
7d	0	15	14	0	30	0	0	0	0	0
7e	23	12	0	20	21	21	0	0	20	11
7f	32	26	27	17	34	32	25	21	30	25
8a	22	18	17	23	22	22	15	21	18	19
8b	20	31	24	16	22	24	10	18	26	26
9	18	15	18	19	22	14	24	19	17	0
10b	28	32	27	22	28	28	30	28	25	30
11b	22	12	10	20	15	15	20	15	13	9
11c	0	0	23	0	12	0	24	0	0	12
11d	32	30	31	25	23	29	20	20	24	32
CFL	20	22	24	25	24	23	20	23	-	-
KET	-	-	-	-	-	-	-	-	23	22

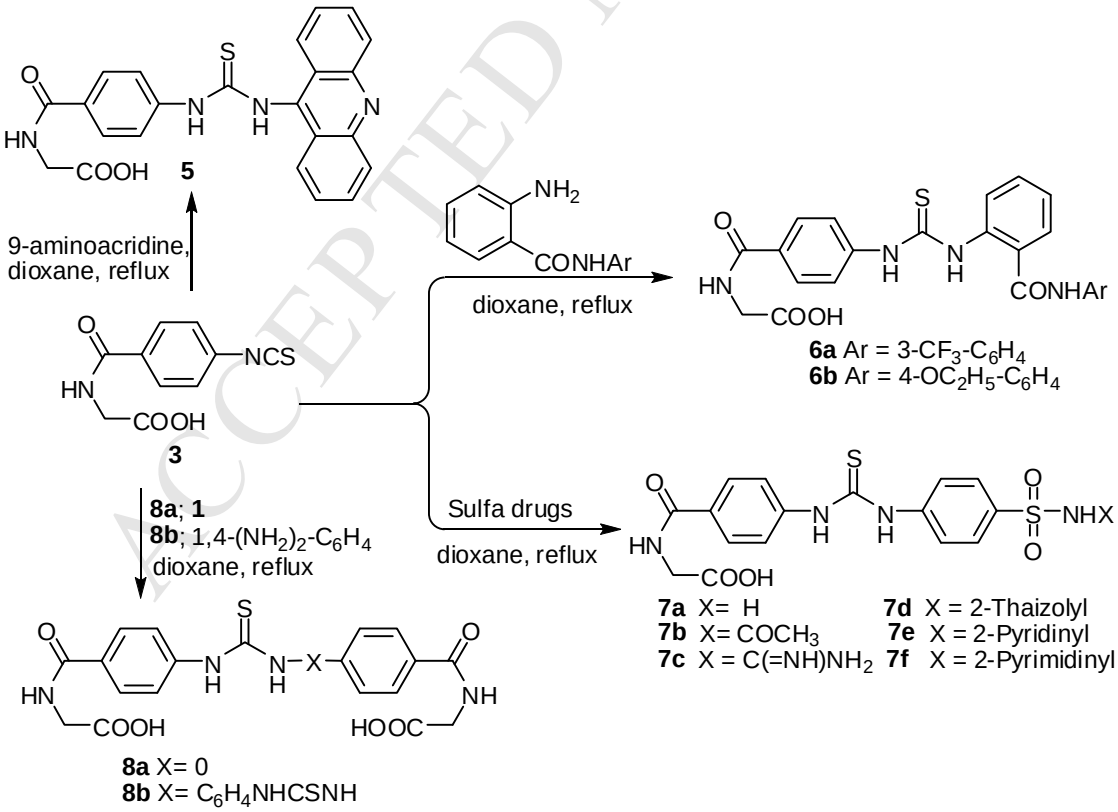
Table 2: Minimum inhibitory concentration (µg/ml) of the more potent synthesized compounds against the pathological organisms

Compd. No.	Gram +ve			Gram -ve					Fungi	
	S. aureus	*B. subtilis*	*B. megaterium*	*K. peneumoniae*	*P. aeroginosa*	*E. coli*	*S. lutea*	*P. vulgaris*	*S. cervesia*	*C. albicans*
4d	6.25	3.12	3.12	6.25	12.5	6.25	12.5	6.25	6.25	3.12
4f	50	12.5	12.5	12.5	12.5	12.5	12.5	12.5	25	12.5
7f	3.12	12.5	12.5	12.5	3.12	6.25	12.5	12.5	3.12	12.5
8a	50	50	12.5	12.5	12.5	12.5	25	25	50	12.5
8b	50	12.5	12.5	12.5	12.5	25	50	25	25	12.5
10b	6.25	3.12	6.25	12.5	12.5	6.25	12.5	6.25	12.5	3.12
11d	6.25	3.12	3.12	12.5	12.5	6.25	12.5	12.5	12.5	3.12
CFL	6.25	6.25	6.25	12.5	12.5	12.5	12.5	12.5	---	---
KET	---	---	---	---	---	---	---	---	3.12	6.25

Scheme 1: Synthesis of mono- and disubstituted thiourea derivatives



Scheme 2: Synthesis of disubstitued thiourea and bisthiourea derivatives



Scheme 3: Synthesis of pyrazolyl thiourea and disubstituted thiosemicarbazide derivatives

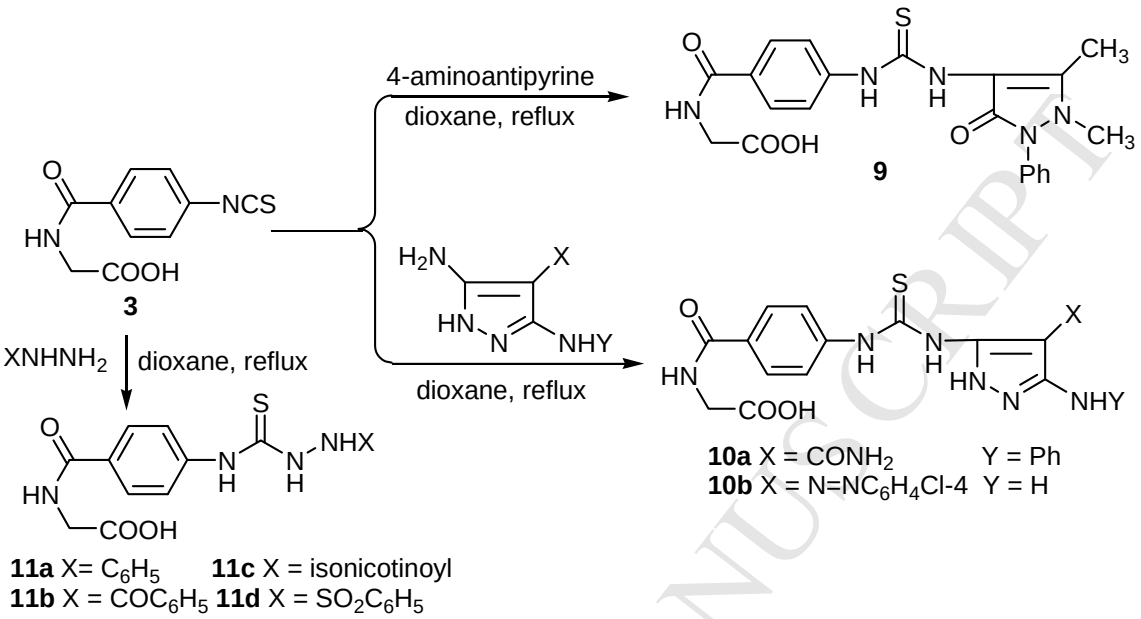


Figure 1

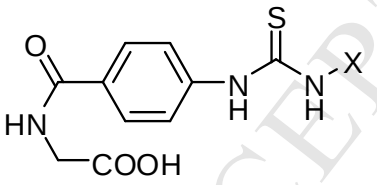


Figure 2: The comparison between the antibacterial activities of our potent synthesized compounds and standard drug against the used Gram positive and Gram negative bacteria.

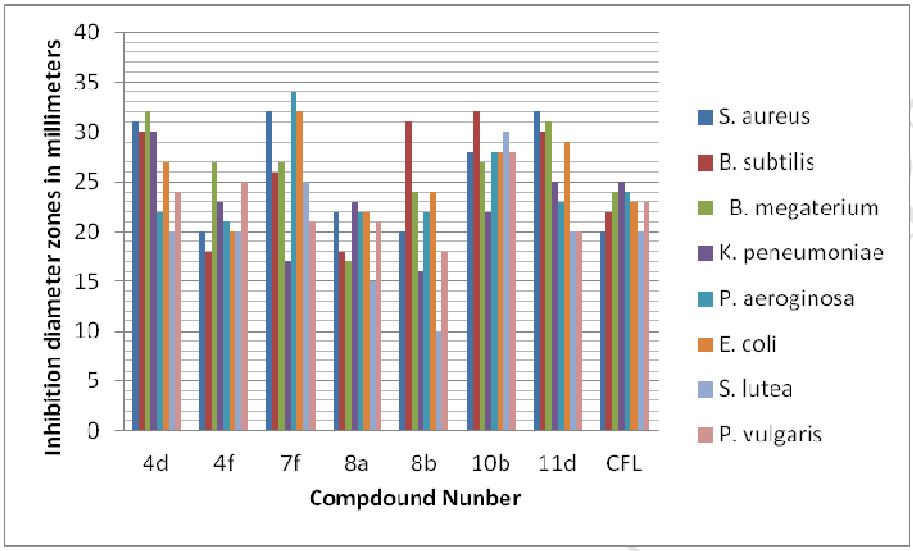


Figure 3: The comparison between the antifungal activities of our potent synthesized compounds and standard drug.