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Synthesis and anti-microbial activity evaluation of some new 1-benzoyl-isothiosemicarbazides

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Abstract

The synthesis of some aroylisothiosemicarbazides was accomplished and their biological activity against bacteria, fungi, and mycobacteria was investigated. Different synthetic pathways were followed according to the kind of substituents that were introduced on both the aroyl ring and the sulfur atom. Anti-bacterial activity was measured against *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus agalactiae* and *S. faecalis*, *Escherichia coli*, and *Salmonella typhi*, while antifungal activity was evaluated against *C. albicans*. Two species, *Mycobacterium tuberculosis* H37RV and *Mycobacterium avium* ATCC19421, were employed to evaluate antimycobacterial activity.

Keywords: Aroylisothiosemicarbazides; Synthesis; Anti-microbial activity

1. Introduction

Thiosemicarbazones (**A**) and thiosemicarbazides (**B**, Fig. 1) are an important class of molecules with a large spectrum of biological properties. These compounds have been studied as anti-tubercular [1–3], anti-bacterial [4–7], anti-malarial [8], anti-leprosy [9], anti-parasitic [10], anti-neoplastic [11–13], and anti-viral [14] agents.

Nevertheless, their derivatives, isothiosemicarbazones (**C**) and isothiosemicarbazides (**D**), have not been studied extensively from a biological point of view.

In a previous investigation on isothiosemicarbazone derivatives an interesting activity against both fungi and bacteria has been shown [15–19]. Moreover, the influence of structural changes, such as, the nature and length of the side chain on the sulphur atom, the introduction of different groups on the arylidene aromatic ring, the substitution of the arylidene hydrogen atom with an alkyl group, the introduction of alkyl groups on the terminal nitrogen atom (N^4), and the replacement of the aromatic ring with heterocyclic rings or cycloalkanes, have been investigated. On the basis of the obtained data some structure–activity relationships have been pointed out.

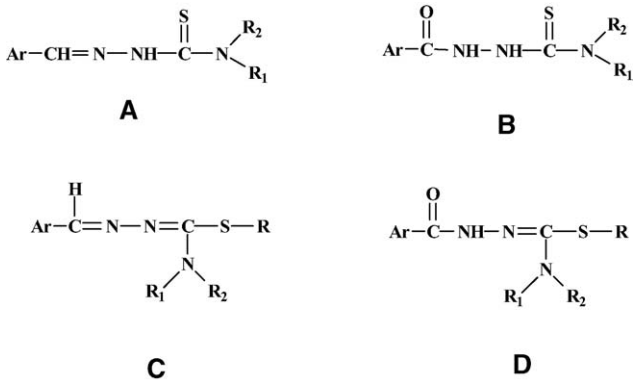

Fig. 1.

Furthermore, the introduction of different substituents on the sulphur atom, standing the previously observed structural requirements, leads to significant changes in the biological properties.

The longer the alkyl chain on the sulphur atom, the higher the activity. However, when the number of carbon atoms is greater than four, a decrease in the biological activity was observed.

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A further increase in the biological activity was observed when the alkyl chain was replaced with benzyl groups. In particular the highest activity was observed when chlorinated benzyl groups were introduced.

On the basis of the previously reported SAR data, a preliminary study on benzoylisothiosemicarbazides **D** has been carried out, in order to investigate their antibacterial and/or antifungal activity.

2. Results and discussion

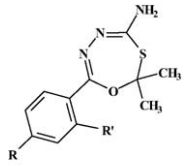
2.1. Chemistry

Isothiosemicarbazides **9–16** (Table 2) have been synthesised by alkylation of the corresponding thiosemicarbazides **1–8** (Table 1).

While slightly modifying the usual methods, three different synthetic pathways shown in Scheme 1 have been followed to optimise the yields of the starting products **1–8** [20–24].

Methods **a** and **c** are similar and consist of the direct benzoylation of the thiosemicarbazides in a base medium, but while method **c** uses pyridine, method **a** uses sodium hydrogen carbonate.

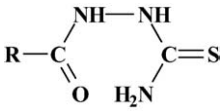
According to method **b** the desired aroylthiosemicarbazides are obtained by reaction of the hydrazides with potassium thiocyanate. Method **a** was used successfully to synthesise benzoylthiosemicarbazide **1**, but it failed when electron withdrawing groups were present in the aromatic ring. In fact when 4-nitrobenzoylchloride was used to achieve 4-nitrobenzoylthiosemicarbazide **6**, with method **a**, a single product was obtained with high yields. Based on analytical and spectral data, the obtained product was compound **17a** and not the expected derivative **6**.



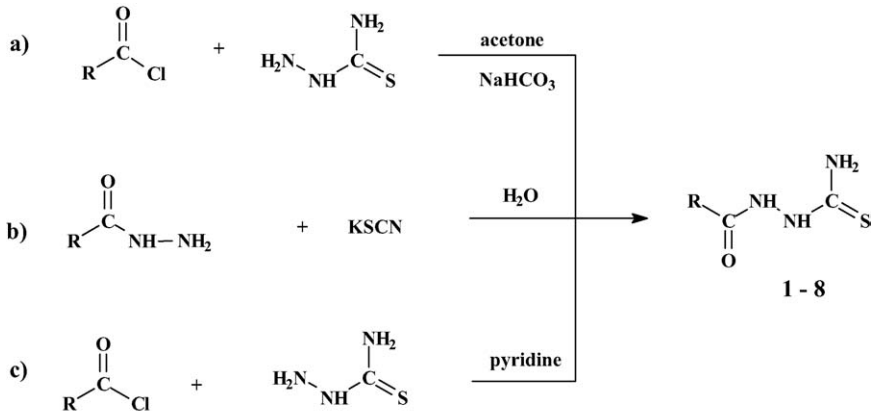
17a: R = NO_2; R'= H
17b: R = Cl ; R'= Cl

A similar behaviour was observed when 2,4-dichlorobenzoylchloride was reacted, though with some dif-

Table 1
Compounds **1–8**



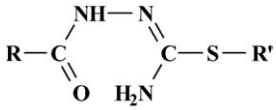
Compound	R	m.p. (°C)	Crystallisation solvent	I.R., cm^{-1} (nujol)
1 [20]	Phenyl	198	Water	3540–3180,1655,1260
2 [20]	4-Methylphenyl	203	Ethanol	3455–3175,1680,1260
3 [20]	4-Chlorophenyl	218–20	Ethanol	3460–3180,1680,1260
4 [20]	4-Methoxyphenyl	236 d	Ethoxyethanol	3390–3130, 1660, 1275, 1250,
5 [22]	2,4-Dichlorophenyl	204	Ethanol	3380–3150,1660,1290
6 [20]	4-Nitrophenyl	216	Ethanol	3500–3140, 1680, 1520, 1340, 1260
7	3,4,5-Trimethoxyphenyl	234	DMF/water	3380–3170, 1650, 1235, 1120
8 [23]	4-Fluorophenyl	172	Ethanol	3430–3130,1660,1270



R = phenyl; 4-methylphenyl; 4-methoxyphenyl; 4-chlorophenyl; 4-nitrophenyl; 4-fluorophenyl; 2,4-dichlorophenyl; 3,4,5-trimethoxyphenyl.

Scheme 1.

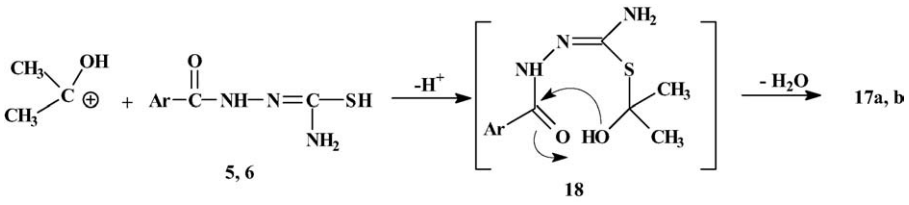
Table 2
Analytical data of synthesized compounds **9–16**



Compound	Formula	m.p. (°C)	Yield %	R	R′	Crystallisation solvent
9a	$C_9H_{11}N_3OS$	231d	85	Phenyl	Methyl	Ethanol/charcoal
9b	$C_{11}H_{13}N_3OS$	235d	80	Phenyl	Allyl	Ethanol/water
9c	$C_{15}H_{14}N_3OSCl$	227d	82	Phenyl	3-Chlorobenzyl	Ethanol/water
9d	$C_{12}H_{17}N_3OS$	230d	63	Phenyl	Butyl	Methanol/water
10a	$C_{10}H_{13}N_3OS$	244d	72	4-methylphenyl	Methyl	Ethyl acetate
10b	$C_{12}H_{15}N_3OS$	252d	91	4-methylphenyl	Allyl	Ethyl acetate
10c	$C_{16}H_{16}N_3OSCl$	248d	77	4-methylphenyl	3-Chlorobenzyl	Ethanol
11a	$C_9H_{10}N_3OSCl$	248d	77	4-chlorophenyl	Methyl	THF/Et.ac. 2:1
11b	$C_{11}H_{12}N_3OSCl$	241d	82	4-chlorophenyl	Allyl	Ethanol/water
11c	$C_{15}H_{13}N_3OSCl_2$	248d	75	4-chlorophenyl	3-Chlorobenzyl	Ethanol/methanol/water
12a	$C_{10}H_{13}N_3O_2S$	244d	83	4-methoxyphenyl	Methyl	Methanol
12b	$C_{12}H_{15}N_3O_2S$	242d	76	4-methoxyphenyl	Allyl	Ethanol/methanol/water
12c	$C_{16}H_{16}N_3O_2SCl$	210d	71	4-methoxyphenyl	3-Chlorobenzyl	Acetonitrile
13a	$C_9H_9N_3OSCl_2$	157-160	70	2,4-dichlorophenyl	Methyl	Ethanol
13b	$C_{11}H_{11}N_3OSCl_2$	205	78	2,4-dichlorophenyl	Allyl	Ethanol/water
13c	$C_{15}H_{12}N_3OSCl_3$	168-170	78	2,4-dichlorophenyl	3-Chlorobenzyl	Ethanol/THF
14a	$C_9H_{10}N_4O_3S$	176d	81	4-nitrophenyl	Methyl	Ethanol
14b	$C_{11}H_{12}N_4O_3S$	238d	80	4-nitrophenyl	Allyl	Methanol
14c	$C_{15}H_{13}N_4O_3SCl$	235d	78	4-nitrophenyl	3-Chlorobenzyl	Methanol
15a	$C_{12}H_{17}N_3O_4S$	223d	47	3,4,5-trimethoxyphenyl	Methyl	Acetonitrile
15b	$C_{14}H_{19}N_3O_4S$	236d	52	3,4,5-trimethoxyphenyl	Allyl	Acetonitrile
15c	$C_{18}H_{20}N_3O_4SCl$	175d	49	3,4,5-trimethoxyphenyl	3-Chlorobenzyl	Methanol/water
16a	$C_9H_{10}N_3OSF$	235d	44	4-fluorophenyl	Methyl	Ethyl acetate
16b	$C_{11}H_{12}N_3OSF$	230d	80	4-fluorophenyl	Allyl	Ethanol/water
16c	$C_{15}H_{13}N_3OSClF$	229d	65	4-fluorophenyl	3-Chlorobenzyl	Ethanol/water

ferences. In this case a mixture of two products was obtained, which after purification were identified as compound **17b** and the desired 2,4-dichlorobenzoylthiosemicarbazide **5**.

The formation of compounds **17a** and **17b** is likely to be due to the weak capability of sodium hydrogen carbonate to behave as a base in a heterogeneous medium. The resulting acidity of the reaction medium could lead to the formation of the intermediate compound **18**, through the reaction of the protonated acetone and aroylthiosemicarbazide. This reaction is favoured by the high electrophilicity of the carbonyl carbon atom, which is due to the presence of electron withdrawing groups in the adjacent aromatic ring.

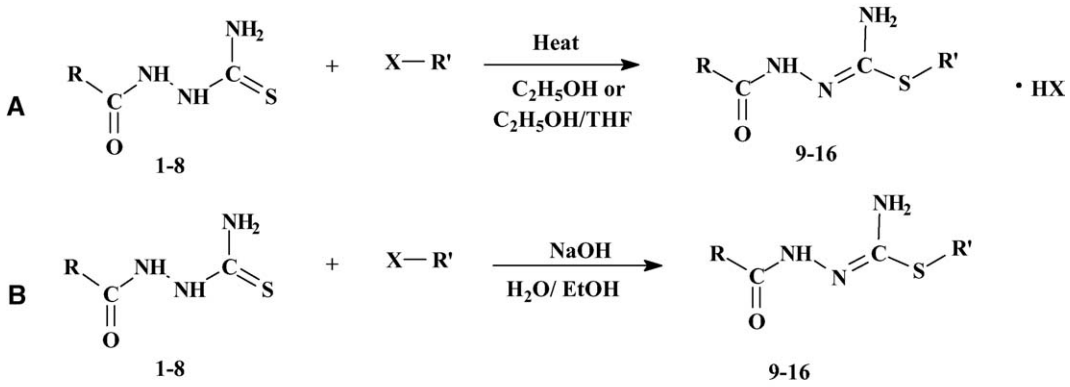


Aroylthiosemicarbazides **2,3,4,7** and **8** were mostly prepared with good yields using method **c**, while 4-nitro- and 2,4-dichloro-benzoylthiosemicarbazides **5** and **6** were prepared using method **b**.

Isothiosemicarbazides **9–16** have been synthesised, with good yields, by alkylation of thiosemicarbazides **1–8** according to two different procedures [25], as depicted in Scheme 2.

All the obtained isothiosemicarbazides have been purified by crystallisation from the appropriate solvent and characterised by elemental analysis, IR, and ^{1}H-NMR.

Only in the case of the 2,4-dichlorobenzoyl derivatives **13a, 13b** and **13c** did ^{1}H-NMR reveal the presence of a double set of signals, probably indicating the presence of two



R = phenyl; 4-methylphenyl; 4-methoxyphenyl; 4-chlorophenyl; 4-nitrophenyl; 4-fluorophenyl; 2,4-dichlorophenyl; 3,4,5-trimethoxyphenyl.
R' = methyl; 3-chlorobenzyl; allyl; n-butyl.

Scheme 2.

geometric isomers on the N=C bond. Any attempt to purify the two diastereoisomers in crystalline form was unsuccessful.

2.2. Microbiology

All the synthesised aroylisothiosemicarbazides were tested against several microbial species to investigate the influence of structural modifications on their antibacterial, antimycobacterial, and antifungal activity.

In particular the antibacterial activity of compounds was measured against four Gram-positive species (*Staphylococcus aureus*, *S. epidermidis*, *Streptococcus agalactiae*, and *S. faecalis*), and two Gram-negative species (*Escherichia coli* and *Salmonella typhi*), while their antifungal activity was evaluated against *C. albicans*.

The antimycobacterial activity was evaluated against two mycobacterial species (Mycobacterium Tuberculosis H37RV, Mycobacterium Avium ATCC19421). All the microorganisms were isolated from clinical specimens.

None of the tested compounds exhibit interesting antibacterial or antifungal activity. Only in the case of compounds **14b** and **15a**, was an activity against *M. tuberculosis* observed at 25 and 50 μg/ml, respectively.

As a matter of fact the replacement of the benzylidene group, previously studied [17], with the benzoyl group leads to a dramatic decrease in antimicrobial activity.

3. Experimental

3.1. Materials and methods

Melting points are uncorrected and were determined on a Reichert Kofler thermopan apparatus. Infrared (I.R.) spectra were recorded on a Perkin–Elmer 1640 FT spectrometer (nujol, cm^{-1}). ^{1}H-NMR spectra were recorded on a Bruker AMX (300 MHz) using tetramethylsilane (TMS) as internal standard and DMSO as solvent (chemical shifts in δ values). Electron ionisation (EI) mass spectra were obtained by a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature 200 °C). The samples were introduced directly into the ion source. Elemental analyses were obtained on a Perkin–Elmer 240 B microanalyser.

3.2. Chemistry

The structures of all compounds were assigned on the basis of IR, NMR, Mass spectra, and elemental analysis.

Analytical data of the synthesised compounds are in agreement with the theoretical data.

3.3. Synthesis of the starting aroylthiosemicarbazides ***1–8***

3.3.1. General methods

(a) Finely pulverised thiosemicarbazide (0.11 mol) and $NaHCO_3$ (9 g) were suspended in dry acetone (155 ml) at –5 °C. The reaction mixture was kept at –5 °C and under vigorous stirring the appropriate benzoyl chloride (0.1 mol) was added dropwise. The mixture was stirred for 4 h at 0 °C, allowed to warm up to room temperature, and then filtered. The solvent was eliminated under vacuum, thus obtaining an oil, which was crystallised from the appropriate solvent.

(b) A mixture of benzoylhydrazide (0.1 mol) and potassium thiocyanate (0.13 mol) was refluxed in water (150 ml) and concentrated hydrochloric acid (85 ml) for 3 h. The mixture was allowed to cool and the formed precipitate was filtered off, washed with water several times, and crystallised from ethanol.

(c) Finely powdered thiosemicarbazide (0.1 mol) was suspended in dry pyridine (100 ml). The reaction mixture was cooled down to –5 °C, and the appropriate benzoylchloride (0.1 mol) added dropwise, keeping temperature

accurately under 0 °C. The reaction was stirred overnight. The crude precipitate was filtered off, washed several times with water, and crystallised.

Compounds 1-(4-methylbenzoyl)thiosemicarbazide **2**, 1-(4-chlorobenzoyl)thiosemicarbazide **3**, 1-(4-methoxybenzoyl)thiosemicarbazide **4**, 1-(3,4,5-trimethoxybenzoyl)-thiosemicarbazide **7**, and 1-(4-fluorobenzoyl)thiosemicarbazide **8** were prepared in good yields using method **c**. Compounds 1-(2,4-dichlorobenzoyl)thiosemicarbazide **5** and 1-(4-nitrobenzoyl)thiosemicarbazide **6** were prepared using method **b**.

*3.3.1.1. Synthesis of benzoxathiadiazepines **17a** and **b***. Using method **a** to react p-nitrobenzoylchloride and thiosemicarbazide, a single product was obtained, which was identified by elemental analysis and with spectroscopics method, as compound **17a**. M.p. 157°C (crystallised from ethanol). I.R. (Nujol): 3440, 3290, 3180 (NH_2), 1590 (C=N), 1510 (NO_2), 1340 (NO_2), cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.15 (s, 6H, CH_3); 6.70 (s, 2H, NH_2, D-exch.); 7.90 (d, 2H, J = 8.8, aromatic protons); 8.40 (d, 2H, J = 8.8, aromatic protons). *m/z*: 280 ($M^{+\cdot}$), 265, 206, 150, 104.

When method **a** was used to achieve 1-(2,4-dichlorobenzoyl)thiosemicarbazide, a mixture of two products was obtained. These two products were separated by crystallisation from acetone and identified as 2,4-dichlorobenzoylthiosemicarbazide **5** and 2,2-dimethyl-4-amino-7-(2,4-dichlorophenyl)-2H-1,3,5,6-oxathiadiazepine **17b**, which was recrystallised from acetone, m.p. 178°C.

I.R. (Nujol): 3380, 3230, 3145 (NH_2), 1600 (C=N), 1515 (Ar), cm^{-1}.

^{1}H-NMR (DMSO-d_6): 2.16 (s, 6H, CH_3), 6.63 (s, 2H, NH_2, D-exch.), 7.35 (d, 1H, J = 6.9, aromatic proton); 7.68 (d, 1H, J = 6.9, aromatic proton); 7.78 (s, 1H, aromatic proton); *m/z*: 304 ($M^{+\cdot}$), 269, 230, 174, 146, 130.

*3.4. Synthesis of Aroylisothiosemicarbazides **9–16***

3.4.1. General methods

(A) 1-aroylthiosemicarbazide (25 mmol) and the appropriate alkyl halides (25 mmol) were refluxed in ethanol (80 ml) for 1 h. The solution was allowed to cool down to room temperature and then ethyl ether was added, thus obtaining the precipitation of isothiosemicarbazide chloride. The obtained solid was treated with a solution of dimethylsulphoxide/water = 1:1 and the obtained solution was treated with $NaHCO_3$ up to the alkaline reaction. The formed isothiosemicarbazide was filtered, washed several times with water, and crystallised from ethanol or a mixture of ethanol/water. We generally perform this reaction to prepare the allylic and benzylic derivative.

(B) Small portions of aroylthiosemicarbazide (30 mmol) were added to an equimolecular solution of sodium hydroxide in ethanol (70 ml). The reaction mixture was stirred for 30 min at room temperature and then alkyl halides (30 mmol) were added. The mixture was stirred at room temperature for a further 30–90 min. The crude reaction was filtered and the solvent evaporated, obtaining a solid, which was washed with water and crystallised.

According to these two procedures isothiosemicarbazides **9–16** were synthesised

*3.4.1.1. 1-benzoyl-S-methylisothiosemicarbazide **9a***. ^{1}H-NMR (DMSO-d_6) δ: 2.48 (s, 3H, CH_3); 6.70 (s, 2H, NH_2, D-exch.); 7.55 (t, 1H, J = 6.8, aromatic proton); 7.59 (t, 1H, J = 7.5, aromatic proton); 7.62 (t, 1H, J = 6.8, aromatic proton); 7.94 (d, 2H, J = 6.8, aromatic protons); 10.11 (s, 1H, CONH, D-exch.).

*3.4.1.2. 1-benzoyl-S-allyl-isothiosemicarbazide **9b***. ^{1}H-NMR (DMSO-d_6) δ: 3.77 (d, 2H, J = 6.7, CH_2S); 5.20 (d, 1H, J = 9.7, CH_2=); 5.41 (d, 1H, J = 17.0, CH_2=); 6.05–6.11 (m, 1H, –CH=); 6.72 (s, 1H, NH_2, D-exch.); 7.54 (t, 1H, J = 6.8, aromatic proton); 7.6 (t, 1H, J = 7.4, aromatic proton); 7.63 (t, 1H, J = 7.6, aromatic proton); 7.94 (d, 2H, J = 6.8, aromatic protons); 10.11 (s, 1H, CONH, D-exch.).

*3.4.1.3. 1-benzoyl-S-(3-chlorobenzyl)isothiosemicarbazide **9c***. ^{1}H-NMR (DMSO-d_6) δ: 4.36 (s, 2H, SCH_2); 6.78 (s, 2H, NH_2, D-exch.); 7.24 (t, 1H, J = 6.6, aromatic proton); 7.29 (d, 1H, J = 7.7, aromatic proton); 7.54 (t, 1H, J = 6.8, aromatic proton); 7.6 (t, 1H, J = 7.4, aromatic proton); 7.61 (d, 1H, J = 6.7, aromatic proton); 7.65 (t, 1H, J = 7.6, aromatic proton); 7.71 (s, 1H, aromatic proton); 7.96 (d, 2H, J = 6.8, aromatic protons); 10.26 (s, 1H, CONH, D-exch.).

*3.4.1.4. 1-benzoyl-S-butyl-isothiosemicarbazide **9d***. ^{1}H-NMR (DMSO-d_6) δ: 1.01 (t, 3H, CH_3); 1.50 (m, 2H, $\underline{CH_2}CH_3$); 1.73 (m, 2H, $\underline{CH_2}CH_2$S); 3.08 (t, 2H, SCH_2); 6.65 (s, 1H, NH_2, D-exch.); 7.53 (t, 1H, J = 6.8, aromatic proton); 7.56 (t, 1H, J = 7.4, aromatic protons); 7.59 (t, 1H, J = 6.8, aromatic proton); 7.92 (d, 2H, J = 7.4, aromatic protons); 10.07 (s, 1H, CONH, D-exch.).

*3.4.1.5. 1-(4-methylbenzoyl)-S-methyl-isothiosemicarbazide **10a***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 6H, SCH_3 and $ArCH_3$); 6.64 (s, 2H, NH_2, D-exch.); 7.34 (d, 2H, J = 7.9, aromatic protons); 7.83 (d, 2H, J = 7.7, aromatic protons); 9.99 (s, 1H, CONH, D-exch.).

*3.4.1.6. 1-(4-methylbenzoyl)-S-allyl-isothiosemicarbazide **10b***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, CH_3); 3.77 (d, 2H, J = 6.7, CH_2S); 5.21 (d, 1H, J = 9.7, CH_2=); 5.41 (d, 1H, J = 17.0, CH_2=); 6.05–6.10 (m, 1H, –CH=); 6.70 (s, 2H, NH_2, D-exch.); 7.36 (d, 2H, J = 7.5, aromatic protons); 7.85 (d, 2H, J = 7.7, aromatic protons); 10.03 (s, 1H, CONH, D-exch.).

*3.4.1.7. 1-(4-methylbenzoyl)-S-(3-chlorobenzyl)isothiosemicarbazide **10c***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, CH_3); 4.36 (s, 2H, CH_2); 6.75 (s, 2H, NH_2, D-exch.); 7.24 (t, 1H,

J = 6.6, aromatic proton); 7.29 (d, 1H, J = 7.7, aromatic proton); 7.39 (d, 2H, J = 7.5, aromatic protons); 7.61 (d, 1H, J = 6.7, aromatic proton); 7.74 (s, 1H, aromatic proton); 7.87 (d, 2H, J = 7.4, aromatic protons); 10.07 (s, 1H, CONH, D-exch.).

3.4.1.8. 1-(4-chlorobenzoyl)-S-methyl-isothiosemicarbazide ***11a***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, CH_3); 6.73 (s, 2H, NH_2, D-exch.); 7.62 (d, 2H, J = 8.4, aromatic protons); 7.97 (d, 2H, J = 8.4, aromatic protons); 10.17 (s, 1H, CONH, D-exch.).

3.4.1.9. 1-(4-chlorobenzoyl)-S-allyl-isothiosemicarbazide ***11b***. ^{1}H-NMR (DMSO-d_6) δ: 3.76 (d, 2H, J = 6.7, CH_2S); 5.19 (d, 1H, J = 9.9, CH_2=); 5.38 (d, 1H, J = 17, CH_2=); 6.05–6.14 (m, 1H, –CH=); 6.79 (s, 2H, NH_2, D-exch.); 7.63 (d, 2H, J = 8.6, aromatic protons); 7.98 (d, 2H, J = 8.4, aromatic protons); 10.19 (s, 1H, CONH, D-exch.).

3.4.1.10. 1-(4-chlorobenzoyl)-S-(3-chlorobenzyl)isothiosemicarbazide ***11c***. ^{1}H-NMR (DMSO-d_6) δ: 4.36 (s, 2H, SCH_2); 6.83 (s, 2H, NH_2, D-exch.); 7.25 (t, 1H, J = 6.7, aromatic proton); 7.29 (d, 1H, J = 7.7, aromatic proton); 7.63 (d, 1H, J =7.4, aromatic protons); 7.73 (s, 1H, aromatic proton), 7.87 (d, 2H, J = 8.6, aromatic protons); 7.98 (d, 2H, J = 8.4, aromatic protons); 10.23 (s, 1H, CONH, D-exch.).

3.4.1.11. 1-(4-methoxybenzoyl)-S-methyl-isothiosemicarbazide ***12a***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, SCH_3); 3.91 (s, 3H, OCH_3); 6.66 (s, 2H, NH_2, D-exch.); 7.08 (d, 2H, J = 8.8, aromatic protons); 7.90 (d, 2H, J = 8.4, aromatic protons); 9.97 (s, 1H, CONH, D-exch.).

3.4.1.12. 1-(4-methoxybenzoyl)-S-allyl-isothiosemicarbazide ***12b***. ^{1}H-NMR (DMSO-d_6) δ: 3.75 (d, 2H, J = 6.5, CH_2S); 3.91 (s, 3H, OCH_3); 5.18 (d, 1H, J = 10.0, CH_2=); 5.40 (d, 1H, J = 16.9, CH_2=); 6.04-6.13 (m, 1H, –CH=); 6.67 (s, 2H, NH_2, D-exch.); 7.07 (d, 2H, J = 8.8, aromatic protons); 7.92 (d, 2H, J = 8.8, aromatic protons); 9.97 (s, 1H, CONH, D-exch.).

3.4.1.13. 1-(4-methoxybenzoyl)-S-(3-chlorobenzyl)isothiosemicarbazide ***12c***. ^{1}H-NMR (DMSO-d_6) δ: 3.93 (s, 3H, OCH_3); 4.34 (s, 2H, SCH_2); 6.71 (s, 2H, NH_2, D-exch.); 7.12 (d, 2H, J = 8.1, aromatic protons); 7.24 (t, 1H, J = 6.6, aromatic proton); 7.29 (d, 1H, J = 7.7, aromatic proton); 7.60 (d, 1H, J = 6.7, aromatic proton); 7.73 (s, 1H, aromatic proton); 7.99 (d, 2H, J = 7.9, aromatic protons); 10.00 (s, 1H, CONH, D-exch.).

3.4.1.14. 1-(2,4-dichlorobenzoyl)-S-methyl-isothiosemicarbazide ***13a***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, SCH_3); 6.61 (s, 2H, NH_2, D-exch.); 7.57 (d, 1H, J = 6.7, aromatic proton); 7.68 (d, 1H, J = 6.7, aromatic proton); 7.87 (s, 1H, aromatic proton); 10.16 (s, 1H, CONH, D-exch.).

3.4.1.15. 1-(2,4-dichlorobenzoyl)-S-allyl-isothiosemicarbazide ***13b***. ^{1}H-NMR (DMSO-d_6) δ: 3.76 (d, 2H, J = 6.9, CH_2S); 5.21 (d, 1H, J = 10.1, CH_2=); 5.41 (d, 1H, J = 17, CH_2=); 6.01–6.14 (m, 1H, –CH=); 6.58 (s, 2H, NH_2, D-exch.); 7.46 (d, 1H, J = 6.8, aromatic proton); 7.63 (d, 1H, J = 6.8, aromatic proton); 7.86 (s, 1H, aromatic proton); 10.17 (s, 1H, CONH, D-exch.).

3.4.1.16. 1-(2,4-dichlorobenzoyl)-S-(3-chlorobenzyl)isothiosemicarbazide ***13c***. ^{1}H-NMR (DMSO-d_6) δ: 4.36 (s, 2H, SCH_2); 6.69 (s, 2H, NH_2, D-exch.); 7.24 (t, 1H, J = 6.6, aromatic proton); 7.29 (d, 1H, J = 7.7, aromatic proton); 7.46 (d, 1H, J = 6.9, aromatic protons); 7.61 (d, 1H, J = 6.7, aromatic proton); 7.64 (d, 1H, J = 6.8, aromatic proton); 7.73 (s, 1H, aromatic proton); 7.86 (s, 1H, aromatic proton); 10.26 (s, 1H, CONH, D-exch.).

3.4.1.17. 1-(4-nitrobenzoyl)-S-methyl-isothiosemicarbazide ***14a***. ^{1}H-NMR (DMSO-d_6) δ: 2.48 (s, 3H, SCH_3); 6.82 (s, 2H, NH_2, D-exch.); 8.19 (d, 2H, J = 8.1, aromatic protons); 8.40 (d, 2H, J = 8.1, aromatic protons); 10.39 (s, 1H, CONH, D-exch.).

3.4.1.18. 1-(4-nitrobenzoyl)-S-allyl-isothiosemicarbazide ***14b***. ^{1}H-NMR (DMSO-d_6) δ: 3.78 (d, 2H, J = 6.5, CH_2S); 5.21 (d, 1H, J = 10.4, CH_2=); 5.41 (d, 1H, J = 16.9, CH_2=); 5.93–6.14 (m, 1H, –CH=); 6.86 (s, 2H, NH_2, D-exch.); 8.18 (d, 2H, J = 7.3, aromatic protons); 8.39 (d, 2H, J = 6.9, aromatic protons); 10.42 (s, 1H, CONH, D-exch.).

3.4.1.19. 1-(4-nitrobenzoyl)-S-(3-chlorobenzyl)isothiosemicarbazide ***14c***. ^{1}H-NMR (DMSO-d_6) δ: 4.39 (s, 2H, SCH_2); 7.28 (s, 2H, NH_2, D-exch.); 7.40 (t, 1H, J = 7.3, aromatic proton); 7.47 (d, 1H, J = 8.1, aromatic proton); 7.59 (d, 1H, J = 7.9, aromatic proton); 7.73 (s, 1H, aromatic proton); 8.21 (d, 2H, J = 8.4, aromatic protons); 8.40 (d, 2H, J = 8.4, aromatic protons); 10.52 (s, 1H, CONH, D-exch.).

3.4.1.20. 1-(3,4,5-trimethoxybenzoyl)-S-methyl-isothiosemicarbazide ***15a***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, SCH_3); 3.81 (s, 3H, OCH_3); 3.94 (s, 6H, OCH_3); 6.67 (s, 2H, NH_2, D-exch.); 7.26 (s, 2H, aromatic protons); 10.03 (s, 1H, CONH, D-exch.).

3.4.1.21. 1-(3,4,5-trimethoxybenzoyl)-S-allyl-isothiosemicarbazide ***15b***. ^{1}H-NMR (DMSO-d_6) δ: 3.76 (d, 2H, J = 6.9, CH_2S); 3.81 (s, 3H, OCH_3); 3.94 (s, 6H, OCH_3); 5.21 (d, 1H, J = 10.1, CH_2=); 5.41 (d, 1H, J = 17.0, CH_2=); 6.06–6.11 (m, 1H, –CH=); 6.71 (s, 2H, NH_2, D-exch.); 7.28 (s, 2H, aromatic protons); 10.06 (s, 1H, CONH, D-exch.).

3.4.1.22. 1-(3,4,5-trimethoxybenzoyl)-S-(3-chlorobenzyl)-isothiosemicarbazide ***15c***. ^{1}H-NMR (DMSO-d_6) δ: 3.82 (s, 3H, OCH_3); 3.95 (s, 6H, OCH_3); 4.53 (s, 2H, CH_2S); 7.17 (s, 2H, NH_2, D-exch.); 7.33 (s, 2H, aromatic protons); 7.40 (t, 1H, J = 7.4, aromatic proton); 7.47 (d, 1H, J = 8.1, aromatic proton); 7.59 (d, 1H, J = 7.4, aromatic proton); 7.70 (s, 1H, aromatic proton); 10.55 (s, 1H, CONH, D-exch.).

3.4.1.23. 1-(4-fluorobenzoyl)-S-methyl-isothiosemicarbazide ***16a***. ^{1}H-NMR (DMSO-d_6) δ: 2.47 (s, 3H, SCH_3); 6.72 (s, 2H, NH_2, D-exch.); 7.36 (t, 2H, J = 8.8, aromatic protons);

8.00 (dt, 2H, J_o = 8.8, J_m = 5.7, aromatic protons); 10.13 (s, 1H, CONH, D-exch.).

3.4.1.24. 1-(4-fluorobenzoyl)-S-allyl-isothiosemicarbazide ***16b***. ^{1}H-NMR (DMSO-d_6) δ: 3.66 (d, 2H, J = 6.8, CH_2S); 5.09 (d, 1H, J = 10.1, CH_2=); 5.29 (d, 1H, J = 17.0, CH_2=); 5.93–5.99 (m, 1H, –CH=); 6.64 (s, 2H, NH_2, D-exch.); 7.25 (t, 2H, J = 8.8, aromatic protons); 7.88 (dt, 2H, J_o = 8.8, J_m = 5.8, aromatic protons); 10.02 (s, 1H, CONH, D-exch.)

3.4.1.25. 1-(4-fluorobenzoyl)-S-(3-chlorobenzyl)-isothiosemicarbazide ***16c***. ^{1}H-NMR (DMSO-d_6) δ: 4.37 (s, 2H, CH_2S); 6.93 (s, 2H, NH_2, D-exch.); 7.26 (t, 2H, J = 8.8, aromatic protons); 7.39 (d, 1H, J = 7.6, aromatic proton); 7.46 (t, 1H, J = 8.1, aromatic proton); 7.59 (d, 1H, J = 7.4, aromatic proton); 7.70 (s, 1H, aromatic proton); 7.88 (dt, 2H, J_o = 8.8, J_m = 5.8, aromatic protons); 10.23 (s, 1H, CONH, D-exch.).

3.5. Microbiology

3.5.1. Compounds

Compounds for antimicrobial studies were dissolved in dimethylsulphoxide at 10 mg/ml and stored at –20 °C. The working solutions were prepared in the same medium as for the tests. To avoid interference with the solvent [26], the highest DMSO concentration was 1%.

3.5.2. Determination of MICs

The MICs of the compounds against Gram-positive bacteria, Gram-negative bacteria, and fungi, were determined by a standard broth macro dilution method [27,28]. Tests with Gram-positive and Gram-negative bacteria were carried out in Mueller Hunton broth (Difco Laboratories, Detroit, MI, USA). Antifungal activity was evaluated in Sabouraud Dextrose broth (Difco Laboratories) [29]. In the case of fungi, MFC (minimum fungicidal concentration) values or more commonly MLC (minimum lethal concentration) values were also measured.

The compounds were diluted in the test medium to obtain a final concentration ranging between 100 and 0.19 μg/ml. Tubes containing 1 ml of the diluted compounds were inoculated with 1×10^5 bacteria and incubated at 37 °C for 18 or 24 h. The determination of MIC against Mycobacteria was carried out by the twofold agar dilution method [30] in multiwell plates (Nunc, Naperville, H, USA) using 7H11 agar (Difco Laboratories) containing the compounds under investigation at concentrations that ranged between 100 and 0.19 μg/ml, on which 100 μl of the test bacterial suspension were spotted.

Suspensions to be used for drug susceptibility testing were prepared from 7H9 broth cultures containing 0.05% Tween 80, washed, suspended in 0.1% Tween 80–saline to yield a no. 1 McFarland turbidity, and then diluted in saline to obtain inocula of $3 \times 10^5 - 1.5 \times 10^4$ cells/100 μl of bacterial suspension. After a cultivation of 21 days (for the slow growers) or of 7 days (for fast growers) in a CO_2 (5% CO_2–95% humidified air) incubator at 37 °C, organism growth was scored. The MIC was defined as the minimum concentration causing complete growth inhibition of organisms or allowing no more than five colonies to grow.

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