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Short communication

Synthesis, characterization and antimicrobial evaluation of ethyl 2-arylhydrazono-3-oxobutyrates

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Abstract – Ethyl 2-arylhydrazono-3-oxobutyrates **6a–f**, 5-(4-aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-one **5d** and 1-[4-(benzoylamino)benzoyl]-4-phenylsemicarbazide **4d** were synthesized in order to determine their antimicrobial properties. Their structures were elucidated using spectral data. The synthesized compounds were tested in vitro against one Gram-positive and 2 Gram-negative bacterial strains, one Mycobacterial strain and a fungus *Candida albicans.* Compound **6d** showed significant activity against *Staphylococcus aureus* whereas the others had no remarkable activity on this strain. Compound **6e** was found to be more active than the others against *Mycobacterium fortuitum* at a MIC value of 32 μg/mL. The antibacterial and antifungal activities of **4d**, **5d** and **6a–f** were also compared with various standard drugs. © Elsevier, Paris

1,2,4-triazoline-3-ones / 1,2,4-triazoline-3-thiones / semicarbazides / coupling products / microdilution method

1. Introduction

A number of compounds derived from hydrazide-hydrazones, 1,2,4-triazoline-3-thiones/1,2,4-triazoline-3-ones and 1,3,4-oxadiazoline-2(3H)-thiones are known to possess antibacterial, antifungal and antimycobacterial activities [1–9]. The coupling products starting from diazonium salts of 4-aminobenzoic acid hydrazide substituted benzalhydrazones with indole have also been reported to possess promising antibacterial and antitubercular activities [10]. In a previous study, 4-(acetylacetonylidenehydrazono)-[(4-fluorophenyl)-methylene]benzoic acid hydrazide were found to be active against *Mycobacterium fortuitum* ATCC 6841 [11]. These observations led us to synthesize some novel coupling products.

In the present study, a new series of ethyl 2-arylhydrazono-3-oxobutyrates **6a**–**f** were obtained via coupling of diazonium chlorides of the above-mentioned heterocyclic rings with ethyl acetoacetate. In addition, the present study includes the synthesis of 1-[4-(benzoylamino)benzoyl]-4-phenylsemicarbazide **4d** and 5-(4-aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-one **5d**. The structures of the synthesized compounds were elucidated by UV, IR, ^{1}H-NMR, ^{13}C-NMR and EI-mass spectral data. These compounds were tested for antimicrobial activities against the microorganisms used in the present study.

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2. Chemistry

4-Aminobenzoic acid [(4-fluorophenyl) methylene]hydrazide **3**; 5-(4-aminophenyl)-2,4-dihydro-4-ethyl/allyl/phenyl-3H-1,2,4-triazole-3-thiones **5a**–**c**; 5-(4-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione **5e** were prepared as described previously [6, 12, 13] *(figure 1)*. 1-[4-(Benzoylamino) benzoyl]-4-phenylsemicarbazide **4d** and 5-(4-aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-one **5d** were determined to be original compounds *(figure 1)*.

These products **3**, **5a**–**e** were then diazotized and coupled with ethyl acetoacetate *(figure 2)*. The coupling products were formulated as ethyl 2-arylhydrazono-3-oxobutyrates **6a**–**f**.

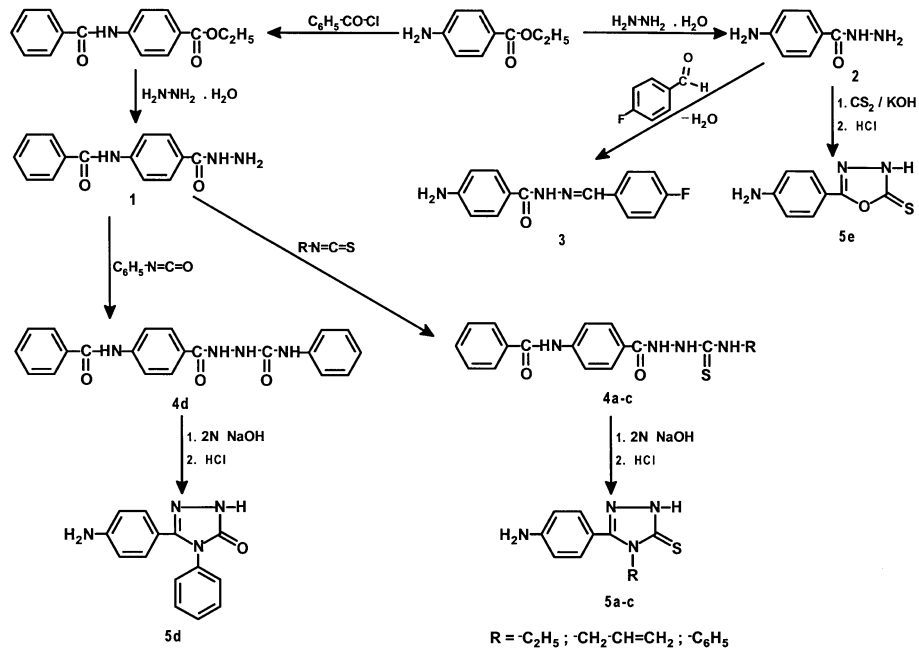

Figure 1. Synthesis of aromatic primary amines.

3. Microbiological results

The synthesized compounds **4d, 5d, 6a–f** were tested for antibacterial, antifungal and antimycobacterial activities against various strains by microdilution method [14–18]. For the determination of antibacterial activity, one Gram-positive and 2 Gram-negative bacterial strains were utilized. Antimycobacterial and antifungal assays of all compounds were also performed against *M. fortuitum* ATCC 6841, a rapidly growing mycobacterium and the yeast *C. albicans* ATCC 2091, respectively.

The results reported in *table I* indicate that, the synthesized compounds were not active against *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 except **6d**, which had a significant growth inhibitory effect on *S. aureus* ATCC 29213 at a MIC value of 7.8 μg/mL. For comparison of the antibacterial activity observed with the test compounds, ceftriaxone was selected as standart drug. The fact that this standart had a reported MIC value of 1–8 μg/mL against *S. aureus* ATCC 29213 showed the significance of further studies on compound **6d**.

Compound **6e** was the most active derivative against *M. fortuitum* ATCC 6841 having the MIC value of 32 μg/mL. This MIC value was also in accordance with that of tobramycine which was 16–32 μg/mL against medium resistant bacteria.

For the observation of the in vitro antifungal activity, the synthesized compounds were screened against *C. albicans* ATCC 2091. However, they were found to have no activity except **5d**, **6c**, **6d**, **6e** and **6f** which showed only marginal effect at a MIC value of 62.5 μg/mL.

4. Discussion

The novel compounds **4d**, **5d**, **6a–f** gave satisfactory elemental analyses. Their structures were established by the use of spectral data (UV, IR, ^{1}H-NMR, ^{13}C-NMR, EI-mass). UV absorption maxima [19], IR stretching and bending vibrations [8] and ^{1}H-NMR chemical shifts [7, 20] were all supported the proposed structures of **4d** and **5d**. ^{13}C-NMR data obtained from **5d** were also in line with literature values [20].

The UV spectra of **6a–f** showed two absorption bands at 235–263 and 360–390 nm regions which were characteristic for carbonyl and hydrazone groups, respectively. In the UV spectra of compounds **6a–f**, absorptions arising from –N=N– band at around 332–360 nm [21, 22] and above 400 nm [23] were not observed. Based on this finding, the structures of these compounds have been

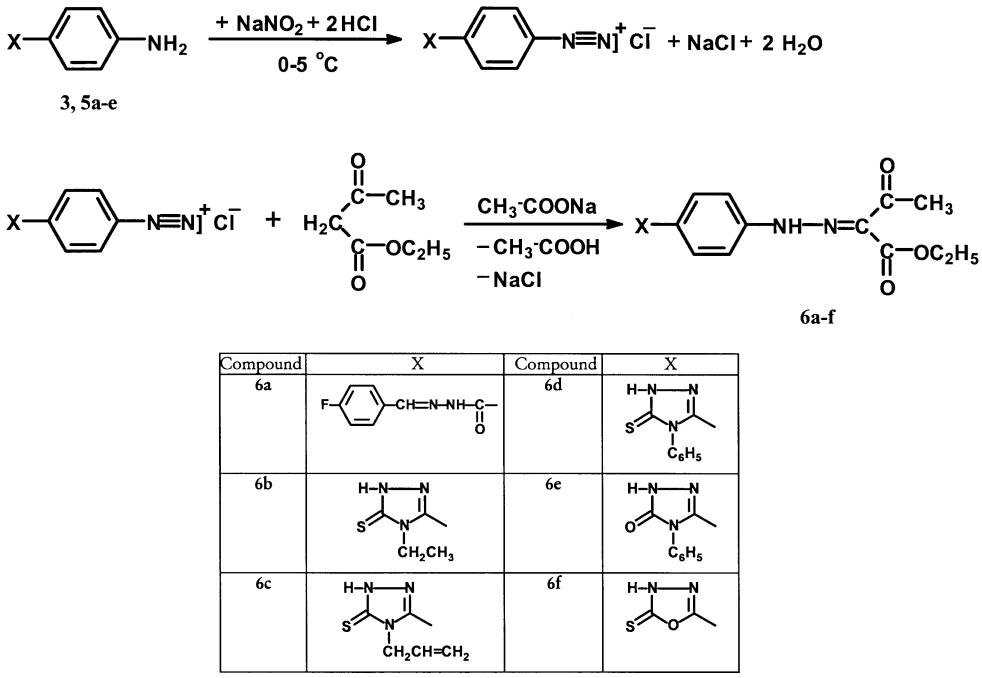

Figure 2. Synthetic route to coupling products **6a–f**.

given in hydrazone form. The UV spectra of **6a**, **6d** and **6f** exhibited the characteristic bands arising from chromophoric –C=N, C=S groups at 319.80, 294.60 and 305.60 nm, respectively.

IR spectra of **6a–f** showed characteristic bands at 1720–1685 and 1690–1660 cm^{-1} corresponding to ester and ketone C=O groups, respectively [24]. N–H strechings due to hydrazone, 1,2,4-triazoline-3-thiones/3-one and 1,3,4-oxadiazoline-2(3H)-thione were detected within the 3400–3150 cm^{-1} region. In addition, the presence of an absorption band arising from triazoline C=S at 1180 cm^{-1} and the lack of absorption bands at around 2590–2250 cm^{-1} supported the thione form of **6b**, **6c**, **6d** and **6f** [6, 25, 26]. The hydrazide–hydrazone C=O and Ar–F bands of **6a** were observed at 1650 and 1170 cm^{-1}, respectively.

The ^{1}H-NMR spectra of **6b** in $CDCl_3$ and **6a**, **6c**, **6d** and **6f** in DMSO-d_6 displayed the hydrazone N–H pro-

Table I. The in vitro antimicrobial activity results of the synthesized compounds (MIC in μg/mL).

Compound	*S. aureus* ATCC 29213	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*M. fortuitum* ATCC 6841	*C. albicans* ATCC 2091
4d	250	125	250	> 128	250
5d	250	250	125	64	62.5
6a	250	250	125	64	125
6b	500	250	125	64	125
6c	250	250	250	128	62.5
6d	7.8	250	125	64	62.5
6e	250	250	125	32	62.5
6f	125	250	125	64	62.5
Ceftriaxone	4	0.03	8	–	–
Tobramycine	–	1	2	16	–
Miconazole	–	–	–	–	0.05

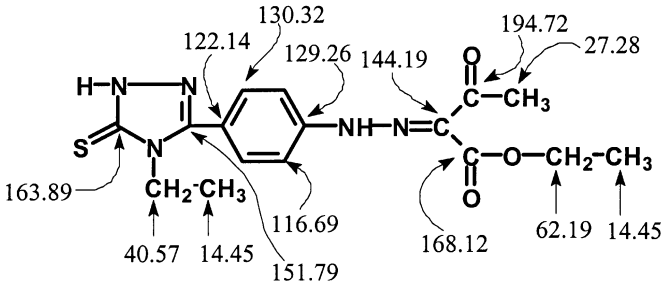

Figure 3. ^{13}C-NMR spectral data of **6b**.

tons at 11.83, 11.84, 11.20, 10.07 and 12.67 ppm, respectively [27]. The hydrazone and triazoline N–H protons of **6e** were observed to exchange with deuterium in DMSO-d_6. In addition, in the ^{1}H-NMR spectra of compounds **6a**–**f**, signals arising from >CH–N=N– structure at 3.00–4.00 ppm [28, 29] were not observed. This finding also supported that the structures of these compounds might be given in hydrazone form.

^{13}C-NMR spectral data of **6b** and **6e** are shown in *figure 3* and *figure 4*, respectively. These results were also in accordance with literature values [26, 30, 31].

EI-mass spectra of **6c**, **6e** and **6f** showed the correct molecular ion (M^+) peaks of different intensity which confirmed their molecular weights. The major fragmentation pathway appeared by the cleavage of –NH–N=C< bonds of hydrazone moiety [26, 32, 33]. Molecular ion was not detected in the mass spectrum of compound **6d** although characteristic fragment ions such as the one at *m/z* 367 (M^+–42), which formed by the loss of CH_2=C=O, were observed. In addition, EI-mass spectra of the synthesized compounds exhibited the expected fragmentation pattern of triazoline and oxadiazoline structures *(figure 5)*.

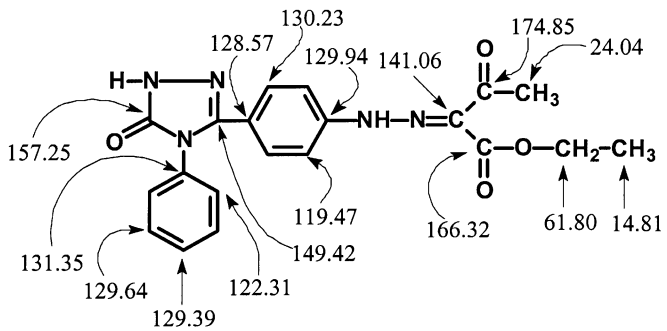

Figure 4. ^{13}C-NMR spectral data of **6e**.

5. Experimental protocols

5.1. Chemistry

Ethyl isothiocyanate, phenyl isothiocyanate and 4-fluorobenzaldehyde were purchased from Sigma. All other chemicals were purchased from Merck.

All melting points were recorded on a Buchi-530 melting point apparatus and uncorrected. UV spectra were recorded on a Shimadzu UV 2100S spectrophotometer (1 mg/100 mL in ethanol). IR spectra were run on a Shimadzu IR-470 spectrophotometer (1 mg/200 mg in KBr). Nuclear Magnetic Resonance spectra (^{1}H, ^{13}C) were recorded on a Bruker AVANC-DPX 400 spectrometer. MS spectra were obtained on a Fisons Instruments VG Platform II LC-MS in the electron impact (EI) mode. Elemental analyses were performed on a Carlo Erba 1106 instrument.

5.1.1. Preparation of aromatic primary amines

These compounds **3**, **5a**–**c**, **5e** were prepared as described previously [6, 12, 13]. In addition, 5-(4-aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-one **5d** having a primary aromatic amine function was synthesized as a novel compound in this study.

*5.1.2. Synthesis of 1-[4-(benzoylamino)benzoyl]-4-phenylsemicarbazide **4d** and 5-(4-aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-one **5d***

4-(Benzoylamino)benzoic acid hydrazide **1** was prepared as described previously [34]. To an ethanolic solution of this compound (0.01 mol) was added phenyl isocyanate (0.015 mol) and the resulting mixture was refluxed for 2 h. The obtained product **4d** was filtered and washed with boiling ethanol. Yield: 83%, m.p. = 270–275 °C. UV (EtOH): λ_{max} (log ε) = 282 (4.43) nm, 235.50 (4.31) nm. IR (KBr): 3450, 3300, 3080, 3050, 1660, 1650, 1600, 1570, 1520, 1440, 1420, 1320, 840, 690 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 6.95–8.13 (m, 14H, Ar-H); 8.84 (s, 1H, –CONHN*H*–); 9.66 (s, 1H, –CON*H*–C_6H_5); 10.19 (s, 1H, C_6H_5–CON*H*–C_6H_4–); 10.41 and 10.47 (2s, 1H, –CON*H*NH–); analysis: $C_{21}H_{18}N_4O_3$ (% calculated/found): 67.37/66.64 (C); 4.84/4.88 (H); 14.96/15.47 (N).

Compound **4d** (0.0134 mol) was refluxed in 65 mL NaOH (2 N) for 4 h. On cooling, it solidified (sodium salt). The precipitate was dissolved in water and acidified with hydrochloric acid (37%) to give compound **5d**. The crude product was washed with water and recrystallized from ethanol. Yield: 30%, m.p. = 274–275 °C. UV (EtOH): λ_{max} (log ε) = 283.50 (4.27) nm. IR (KBr): 3410, 3350, 3190, 3050, 1695, 1630, 1615, 1580, 1510, 1460,

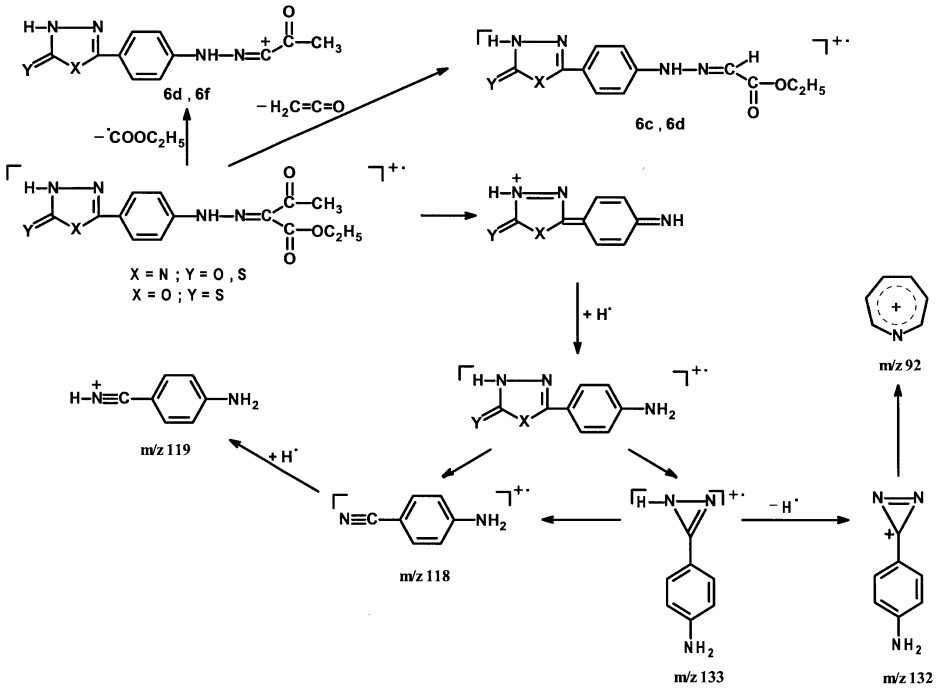

Figure 5. Mass spectral fragmentation pattern of compounds **6c–f**.

1440, 1280, 830, 710 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 5.36 (s, 2H, Ar–NH_2); 6.42 (d, 2H, o-NH_2, *J* = 8 Hz); 6.91 (d, 2H, m-NH_2, *J* = 8 Hz); 7.21–7.46 (m, 5H, Ar–H); 11.78 (s, 1H, triazoline N–H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ ppm: 114.01 (C2 and C6); 114.64 (C8 and C12); 128.63 (C4); 129.10 (C10); 129.49 (C9 and C11); 130.03 (C3 and C5); 135.08 (C7); 147.04 (C1); 151.02 (triazoline, C=N); 155.51 (oxolactam, C=O); analysis $C_{14}H_{12}N_4O$ (% calculated/found): 66.65/66.93 (C); 4.80/4.74 (H); 22.21/22.26 (N).

5.1.3. Synthesis of ethyl 2-arylhydrazono-3-oxobutyrates ***6a–f***

Aryldiazonium salts of the aromatic primary amines **3, 5a**–**e** were formed by the action of nitrous acid on compounds **3**, **5a**–**e** at 0–5 °C. These were then coupled with ethyl acetoacetate in the presence of sodium acetate.

5.1.4. Ethyl 2-{4-[[(4-fluorophenyl)methylene]hydrazinocarbonyl]phenylhydrazono}-3-oxobutyrate ***6a***

Yield: 58%; m.p. = 171–175 °C. UV (EtOH): λ_{max} (log ε) = 319.80 (4.60) nm. IR (KBr) 3200, 3080, 3040, 2910, 1720, 1690, 1650, 1600, 1570, 1510, 1420, 1380, 1170, 850, 840 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 1.28 (t, 3H, –O–CH_2–CH_3); 2.42 (s, 3H, –COCH_3); 4.33 (q, 2H, –O–CH_2–CH_3); 7.27–8.00 (m, 8H, Ar–H); 8.68 (s, 1H, –CH=N–); 11.41 (s, 1H,=N–NH–CO–); 11.84 (s, 1H, –NH–N=C<); analysis $C_{20}H_{19}FN_4O_4$ (% calculated/found): 60.30/61.91 (C); 4.81/4.53 (H); 14.06/13.59 (N).

5.1.5. Ethyl 2-[4-(2,4-dihydro-4-ethyl-3H-1,2,4-triazole-3-thione-5-yl)phenylhydrazono]-3-oxobutyrate ***6b***

Yield: 95%; m.p. = 170–172 °C. UV (EtOH): λ_{max} (log ε) = 362 (4.43) nm, 256.80 (4.33) nm. IR (KBr): 3190, 3000, 2950, 1685, 1660, 1610, 1520, 1480, 1460, 1440, 1320, 1280, 1180, 845 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 1.41 (m, 6H, –O–CH_2–CH_3 and –N–CH_2–CH_3); 2.54 (s, 3H, –CO–CH_3); 4.20 (q, 2H, –N–CH_2–CH_3); 4.42 (q, 2H, –O–CH_2–CH_3); 7.50 (d, 2H, o-NH, *J* = 8.5 Hz); 7.65 (d, 2H, m-NH, *J* = 8.5 Hz); 11.83 (b, 1H, –NH–N=C<); 12.76 (s, 1H, triazoline N–H). ^{13}C-NMR (100.6 MHz, $CDCl_3$) δ ppm: 14.45 (m, –O–CH_2–CH_3 and –N–CH_2–CH_3); 27.28 (–CO–CH_3); 40.57 (–N–CH_2–CH_3); 62.19 (–O–CH_2–CH_3); 116.69 (C2 and C6); 122.14 (C4); 129.26 (C1); 130.32 (C3 and C5); 144.19 (hydrazone, C=N); 151.79 (triazoline, C=N); 163.89 (thiolactam, C=S); 168.12 (ester, –COO–C_2H_5); 194.72 (ketone, –CO–CH_3); analysis $C_{16}H_{19}N_5O_3S$ (%

calculated/found): 53.17/52.88 (C); 5.30/5.22 (H); 19.38/19.08 (N); 8.87/8.32 (S).

5.1.6. Ethyl 2-[4-(4-allyl-2,4-dihydro-3H-1,2,4-triazole-3-thione-5-yl)phenylhydrazono]-3-oxobutyrate ***6c***

Yield: 98%; m.p. = 85–90 °C. UV (EtOH): λ_{max} (log ε) = 378.60 (4.26) nm, 258.60 (4.17) nm. IR (KBr): 3450–3350, 3150, 2900, 1710, 1660, 1610, 1590, 1540, 1480, 1250, 1180, 840 cm^{-1}. EI-MS (*m/z*): 373 (M^+), 341, 332, 331, 326, 286, 260, 258, 246, 232, 231, 217, 216, 157, 133, 132, 119, 118, 92. ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 1.18 (t, 3H, –O–CH_2–CH_3); 2.19 (s, 3H, –CO–CH_3); 4.18 (q, 2H, –O–CH_2–CH_3); 4.90 (d, 1H, allyl, =C<^{H} trans, *J* = 16 Hz); 5.15 (d, 1H, allyl, =C<$_H$ cis, *J* = 10 Hz); 5.82–6.10 (m, 1H, allyl, –CH =); 7.56 (d, 2H, o-NH, *J* = 8.6 Hz); 7.73 (d, 2H, m-NH, *J* = 8.6 Hz); 11.20 (s, 1H, –NH–N=C<); 13.98 (s, 1H, triazoline N–H): analysis for $C_{17}H_{19}N_5O_3S•H_2O$ (% calculated/found): 52.16/51.85 (C); 5.41/4.85 (H); 8.19/8.05 (S).

5.1.7. Ethyl 2-[4-(2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-thione-5-yl)phenylhydrazono]-3-oxobutyrate ***6d***

Yield: 89%; m.p. 205–210 °C. UV (EtOH): λ_{max} (log ε) = 390 (3.65) nm, 294.60 (4.45) nm, 263 (4.37) nm. IR (KBr): 3400, 3150, 3080, 2900, 1720, 1650, 1610, 1590, 1550, 1500, 1420, 1320, 1250, 1180, 840, 700 cm^{-1}. EI-MS (*m/z*): 367 (M^+–42), 337, 269, 268 (100%), 267, 150, 133, 132, 119, 118, 92, 77, 65, 63. ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 1.89 (m, 3H, –O–CH_2–CH_3); 2.03 (s, 3H, –CO–CH_3); 4.11 (q, 2H, –O–CH_2–CH_3); 7.29–7.51 (m, 9H, Ar–H); 10.07 (s, 1/2H, –NH–N=C<); 13.36 (b, 1H, triazoline N–H): analysis $C_{20}H_{19}N_5O_3S$ (% calculated/found): 58.67/58.05 (C); 4.68/4.70 (H); 17.11/17.83 (N).

5.1.8. Ethyl 2-[4-(2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-one-5-yl)phenylhydrazono]-3-oxobutyrate ***6e***

Yield: 80%; m.p. 235–238 °C. UV (EtOH): λ_{max} (log ε) = 360 (4.05) nm; 239.40 (4.36) nm. IR (KBr): 3500–3400, 3300, 3200, 3100, 2900, 1680, 1600, 1550, 1500, 1440, 1280, 840, 700 cm^{-1}. EI-MS (*m/z*): 393 (M^+) 306, 278, 261, 259, 253, 252, 251, 177, 175, 134, 133, 120, 119, 118, 92, 77, 63. ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 1.28 (t, 3H, –O–CH_2–CH_3); 1.80 (s, 3H, –CO–CH_3); 4.27 (m, 2H, –O–CH_2–CH_3); 7.24–7.98 (m, 9H, Ar–H).^{13}C-NMR (100.6 MHz, DMSO-d_6): δ ppm 14.81 (–O–CH_2–*C*H_3); 24.04 (–CO–*C*H_3); 61.80 (–O–*C*H_2–CH_3); 119.47 (C2 and C6); 122.31 (C8 and C12); 128.57 (C4); 129.39 (C10); 129.64 (C9 and C11);129.94 (C1); 130.23 (C3 and C5); 131.35 (C7); 141.06 (hydrazone, C=N); 149.42 (triazoline, C=N); 157.25 (oxolactam, C=O); 166.32 (ester, –*C*OO–C_2H_5); 174.85 (ketone, –*C*O–CH_3): analysis $C_{20}H_{19}N_5O_4•3H_2O$ (% calculated/found): 53.69/53.21 (C); 5.63/4.74 (H); 15.65/15.35 (N).

5.1.9. Ethyl 2-[4-(1,3,4-oxadiazole-2(3H)-thione-5-yl) phenylhydrazono]-3-oxobutyrate ***6f***

Yield: 80%; m.p. = 178–180 °C. UV (EtOH): λ_{max} (log ε) = 383.60 (4.40) nm; 305.60 (4.16) nm; 235 (4.17) nm. IR (KBr): 3450–3350, 3100, 2950, 2800, 1680, 1610, 1580, 1510, 1500, 1180, 840 cm^{-1}. EI-MS (*m/z*): 336, 335, 334 (M^+), 290, 289, 262, 261, 246, 219, 194, 193, 192, 187, 158, 157, 133, 132 (100%), 92, 65, 63. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 1.38 (t, 3H, –O–CH_2–CH_3); 2.49 (s, 3H, –CO–CH_3); 4.36 (m, 2H, –O–CH_2–CH_3); 7.40 (d, 2H, o-NH, *J* = 8.6 Hz); 7.93 (d, 2H, m-NH, *J* = 8.6 Hz); 12.67 (s, 1H, –NH–N=C<); 14.60 (s, 1/2H, oxadiazoline N–H): analysis $C_{14}H_{14}N_4O_4S•H_2O$ (% calculated/found): 47.72/47.52 (C); 4.58/3.85 (H); 15.90/16.03 (N); 9.10/10.01 (S).

5.2. Microbiology

Compounds **4d**, **5d** and **6a–f** were examined for their in vitro growth inhibitory activity against different bacterial strains in addition to *M. fortuitum* ATCC 6841, a rapidly growing mycobacterium and a yeast-like fungus, *C. albicans* ATCC 2091. Bacterial strains utilized were *S. aureus* ATCC 29213 as Gram-positive; *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 as Gram-negative bacteria. Antibacterial, antifungal and antimycobacterial assays were all performed by the use of the two-fold serial microdilution technique [14–18].

5.2.1. Antibacterial and antifungal activity

Standardized bacterial and fungal inocula were prepared by touching the top of four or five colonies of a single type and inoculating them into a tube containing 5 mL of Mueller–Hinton broth (Difco) at pH 7.3 for bacteria and buffered Yeast Nitrogen Base (YNB) at pH 7 for *C. albicans*. Incubations of these microorganism suspensions were carried out at 35 °C for bacteria and the yeast *C. albicans* until a visible turbidity was obtained. The density of these cultures were then adjusted to a turbidity equivalent to that of a 0.5 Mc Farland standard and finally, the adjusted culture was diluted so that, after inoculation, each microplate well had an inoculum size of 5×10^5 cfu/mL for bacteria and 0.5×10^3 to 2.5×10^3 cells per mL for *C. albicans*.

Antibacterial and antifungal assays were performed in Mueller–Hinton broth at pH 7.3 and buffered Yeast Nitrogen Base at pH 7, respectively. Ceftriaxone for bacteria and Miconazole for the yeast *C. albicans* were used as standard drugs. All the test compounds were dissolved in DMSO. Further dilutions of the compounds

and the standard drugs in the test medium were furnished at the required quantities of the broth used. The concentration range was 32–0.015 μg/mL for Ceftriaxone, 100–0.05 μg/mL for Miconazole and 128–1 μg/mL for the test compounds. After inclusion of 100 μL of the broth containing the standard drugs or the test compounds, 100 μL of bacterial or fungal suspension were inoculated into microplate wells. After incubation for 16–20 h at 35 °C (for bacteria) or 46–50 h at 35 °C (for *C. albicans*), the well, containing the lowest concentration of the standard drugs or the test compounds that inhibits microorganism growth as detected by the unaided eye, was recorded to represent the MIC expressed in μg/mL.

5.2.2. Antimycobacterial activity

Preparation of Mycobacterial inoculum required a few modifications due to the difficulty on obtaining a homogenous suspension of *M. fortuitum* in the broth used. Four or five colonies of *M. fortuitum* which were previously grown in Tryptic Soy Agar (TSA) after 72 h of incubation at 30 °C were collected by means of a swab and suspended in 4.5 mL of Mueller–Hinton broth enriched with Tween 80 (0.2%). Following the inclusion of 4–5 glass beads, this mixture was whirled using a vortex-mixer to ensure a good suspension. The density of this culture was then adjusted to a turbidity equivalent to that of a 0.5 Mc Farland standard and finally, the adjusted culture was diluted with sterile water so that, after inoculation, each microplate well had an inoculum size of 1.5×10^5 cfu/mL.

Antimycobacterial testing of all compounds was carried out in Mueller–Hinton broth enriched with Tween 80 (0.2%) at pH 7.3. Tobramycine which is an active antibiotic against rapidly growing mycobacteria was selected as standard drug. Quality control strains used in the present study were *E. coli* and *S. aureus*. Tobramycine was used at a concentration range of 4–0.03 μg/mL against the quality control strains. The standard drug and test compounds were dissolved in water and DMSO, respectively, and were diluted with the broth used. The concentration intervals were 32–0.5 μg/mL and 128–1 μg/mL for the standard drug and the test compounds, respectively. Microplate wells, added with 100 μL of broth containing Tobramycine or the test compounds, were then inoculated with 10 μL of *M. fortuitum* suspension whose preparation were mentioned above. Sheep-blooded agar was used for the purity control. After incubation for 72 h at 30 °C, the last microplate well with no growth of microorganism was recorded to represent MIC expressed in μg/mL.

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