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Short communication

Synthesis, characterization and pharmacological properties of some 4-arylhydrazono-2-pyrazoline-5-one derivatives obtained from heterocyclic amines

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Abstract – The synthesis of a new series of 4-arylhydrazono-2-pyrazoline-5-ones **7–24** and **22a** is described. Structures of the synthesized compounds were confirmed using UV, IR, ^{1}H-NMR, ^{13}C-NMR and EI-mass spectral data. These compounds were tested in vitro against one Gram-positive and two Gram-negative bacterial strains, two mycobacterial strains and a fungus, *Candida albicans*. Compound **22** was found to be more active against *Staphylococcus aureus* than the other compounds at a concentration of 15.6 µg/mL. Some related compounds were evaluated for anticonvulsant activity. Compound **11** showed 40% protection against pentylenetetrazole-induced seizures in albino Swiss mice. In vitro antituberculosis activity of 4-arylhydrazono-2-pyrazoline-5-ones **7–12**, **14–24**, **22a** and coupling products **6a–f** were tested on *Mycobacterium tuberculosis* H37Rv. Of these compounds, only **24**, which exhibited > 90% inhibition in the primary screen at 12.5 µg/mL against this strain was re-examined for determination of its actual MIC. However, level II assay revealed that the MIC value was not less than 12.5 µg/mL. The same compound was also tested against *Mycobacterium avium*, which was observed not to be susceptible to **24**. © 2000 Éditions scientifiques et médicales Elsevier SAS

4-arylhydrazono-2-pyrazoline-5-one / BACTEC 460 radiometric system / antimicrobial activity / anticonvulsant activity / reductive cleavage

1. Introduction

Some compounds bearing the pharmacophoric group shown in *figure 1* have been reported to be responsible for antibacterial [1–3], antifungal [3–5] and anticonvulsant activities [5, 6].

On the other hand, hydrazide-hydrazones [7–10], 1,2,4-triazoline-3-thiones [11, 12], 1,2,4-triazoline-3-ones [13–15] and 1,3,4-oxadiazoline-2(3H)-thiones [16, 17] are known to be responsible for a wide variety of pharmacological effects. These observations led us to synthesize some novel compounds via combination of a 2-pyrazoline ring with one of the above mentioned heterocyclic rings linked by a phenylhydrazono moiety and to investigate their possible pharmacological activities.

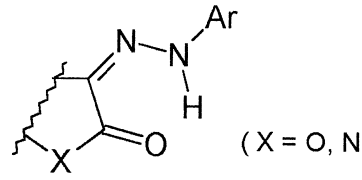

Figure 1. Proposed pharmacophore responsible for antibacterial, antifungal or anticonvulsant activities.

In the present study, ethyl 2-arylhydrazono-3-oxobutyrates which were previously reported [18], were reacted with substituted hydrazines and some novel

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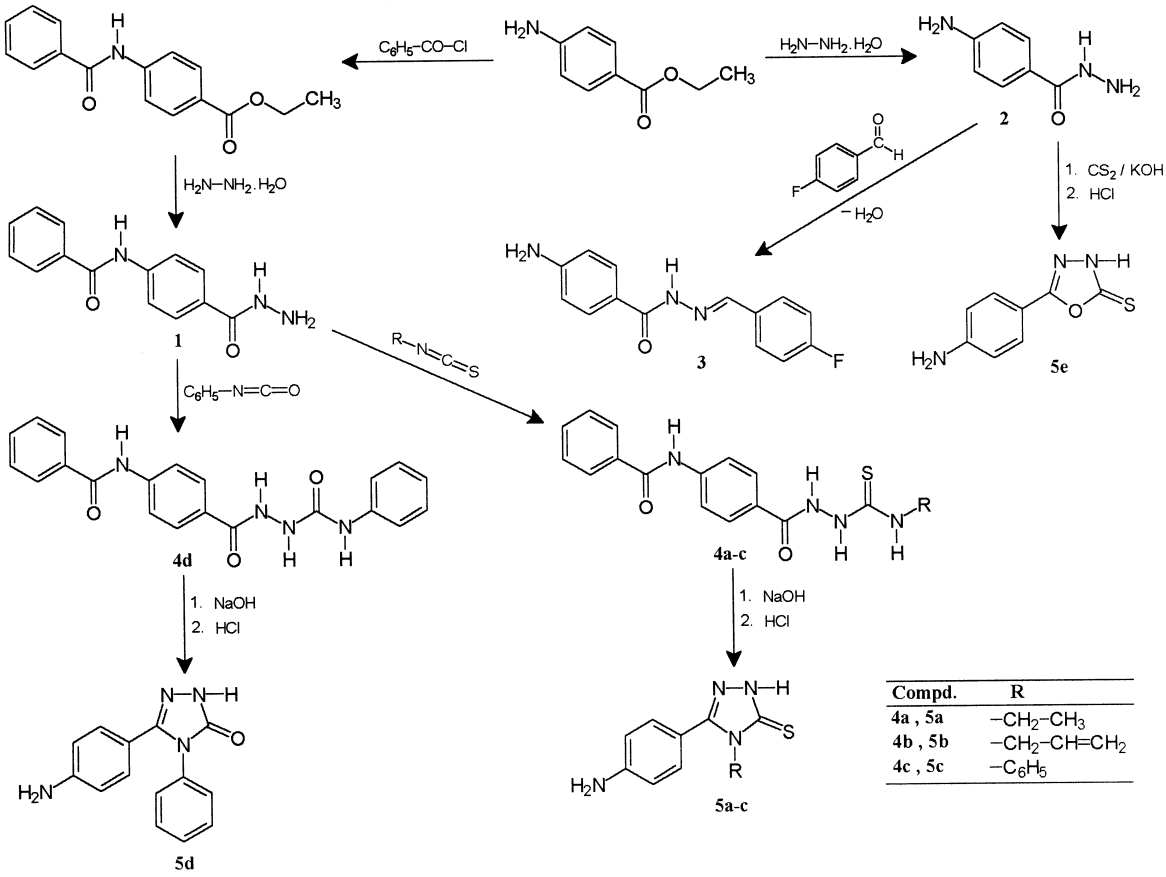


Figure 2. Synthesis of the primary aromatic amines **3** and **5a–e**.

4-arylhydrazono-2-pyrazoline-5-one derivatives were synthesized. Structures of the synthesized compounds were elucidated by the use of UV, IR, ^{1}H-NMR, ^{13}C-NMR and EI-mass spectral techniques.

Finally, the synthesized compounds were tested for their antibacterial, antifungal and antimycobacterial activities against various strains. Some selected compounds were evaluated for anticonvulsant activity against pentylenetetrazole induced seizures in male albino Swiss mice and for antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv and *Mycobacterium avium.*

2. Chemistry

Compounds containing an aromatic primary amine function were prepared as described previously [12, 18–20] (*figure 2*). These products were then diazotized and coupled with ethyl acetoacetate to give **6a–f**. The structures of the coupling products **6a–f** were formulated as ethyl 2-arylhydrazono-3-oxobutyrates [18]. As the final step, the coupling products **6a–f** were reacted with substituted hydrazines in glacial acetic acid medium to give some novel 4-arylhydrazono-2-pyrazoline-5-one derivatives (*figure 3*).

Compound **22a** was synthesized by refluxing compound **22** in acetic anhydride. 5-(4-Aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-thione **22b** was obtained by the reductive cleavage of compound **22** with hydrazine-hydrate according to the method described in the literature [21, 22] (*figure 4*).

The synthesized compounds were characterized by their combustion analyses, melting points and TLC analyses on silicagel. The structures of novel compounds **7–24** and **22a** were established using UV, IR, ^{1}H-NMR, ^{13}C-NMR and EI-mass spectral techniques. These physical and spectral data of the compounds **7–24** and **22a** are presented in *tables I* and *II*.

UV spectra of the cyclization products **7–24** exhibited the bands arising from a hydrazone (-NH–N=C<) group at 432–377 nm and a carbonyl (>C=O) function at

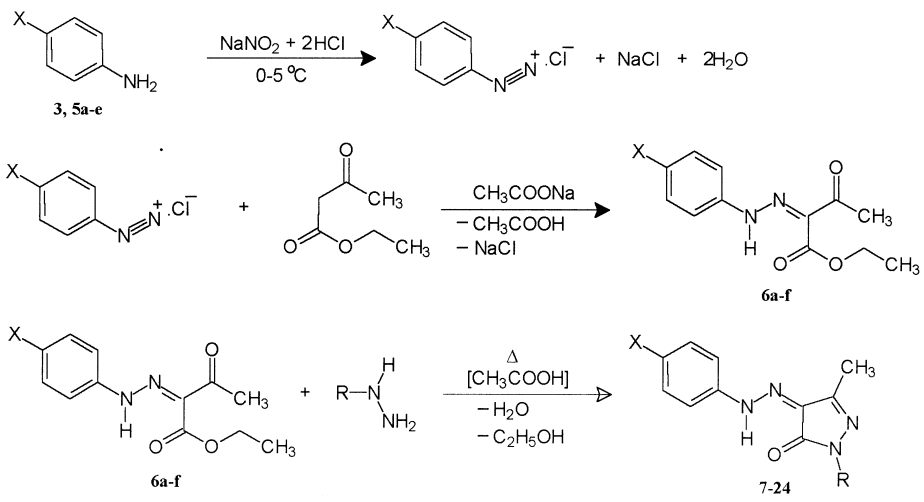
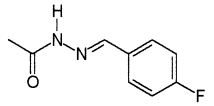
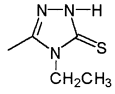
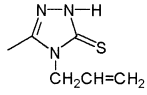
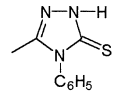
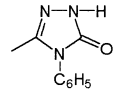
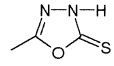


Figure 3. Synthesis of 4-arylhydrazono-2-pyrazoline-5-one derivatives **7–24**.

Compd.	R	X
6a	—	
7	-H	
13	$-CH_2-CH_2-OH$	
19	$-C_6H_5$	
6b	—	
8	-H	
14	$-CH_2-CH_2-OH$	
20	$-C_6H_5$	
6c	—	
9	-H	
15	$-CH_2-CH_2-OH$	
21	$-C_6H_5$	

Compd.	R	X
6d	—	
10	-H	
16	$-CH_2-CH_2-OH$	
22	$-C_6H_5$	
6e	—	
11	-H	
17	$-CH_2-CH_2-OH$	
23	$-C_6H_5$	
6f	—	
12	-H	
18	$-CH_2-CH_2-OH$	
24	$-C_6H_5$	

267–235 nm. These compounds showed no absorption at above 400 nm [23] and at 332–360 nm showing the lack of azo structure [24, 25]. The observation of bands at around 432–377 nm indicated that compounds **7–24** were in the hydrazone form [26].

The IR spectra of compounds **7–24** exhibited >C=O bands at the 1 680–1 650 cm^{-1} region as a confirmation of common 2-pyrazoline-5-one structures [27, 28]. The compounds containing 1,2,4-triazoline-3-thione, 1,3,4-oxadiazoline-2(3H)-thione and hydrazide-hydrazone moieties displayed thione (>C=S) and carbonyl (>C=O) vibrations at 1 180 cm^{-1} and 1 650–1 640 cm^{-1}, respectively. The -OH stretching vibration bands of compounds **13–18**, which were obtained by the reaction of compounds **6a–f** with β-hydroxyethylhydrazine, were detected at 3 500–3 350 cm^{-1}. The absorption bands of other functional groups also appeared in the expected regions.

^{1}H-NMR spectra of the cyclization products **7–24** displayed the resonances of hydrazone N–H at 11.59–13.64 ppm and methyl protons at 2.12–2.35 ppm. The hydrazone N–H structure of **7–24** was also supported by ^{1}H-NMR spectra. Any signals which were attributable to the probable structure of >CH–N=N- were not shown at 3.00–4.00 ppm [29, 30]. ^{1}H-NMR data also proved the existence of *Z* and *E* forms of compounds **14** and **15** [31, 32]. Azomethine protons of compounds **7**, **13** and **19** resonated at 9.83, 8.47 and 9.85 ppm, respectively [33, 34]. The hydrazone, triazoline and pyrazoline N–H protons of compound **8** were observed to exchange with D_2O in the ^{1}H-NMR spectrum. The triazoline, oxadiazoline and hydrazone N–H protons and hydroxyl protons of some compounds exchanged with deuterium in the solvent used for obtaining the ^{1}H-NMR spectra. Resonances of pyrazoline N–H protons of compounds **7–12**, which were obtained by the reaction of compounds **6a–f** with

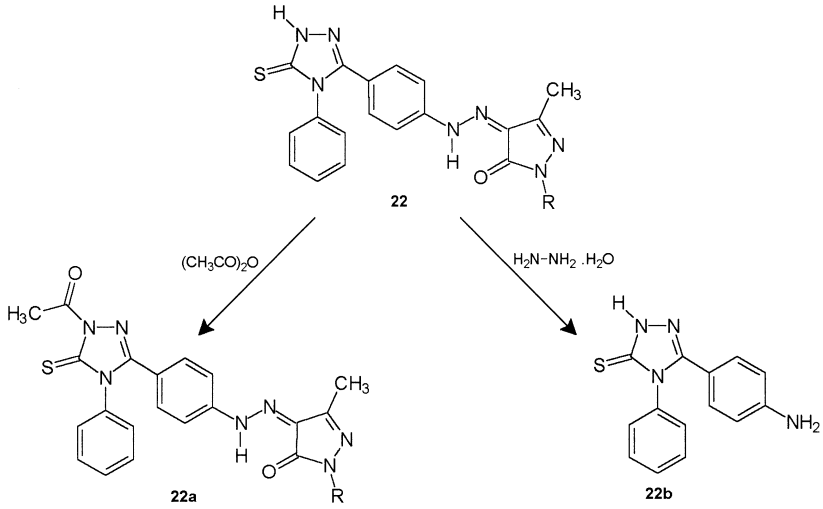


Figure 4. Acetylation and reductive cleavage of compound **22**.

hydrazine hydrate were observed at 10.54–11.59 ppm (as singlets). The hydroxylic proton of compounds **13–15**, **17** and **18** resonated as singlets at 4.75–4.78 ppm, whereas compound **16** exhibited this signal at 3.88 ppm [35, 36].

^{13}C-NMR spectral data of compounds **12**, **16** and **21** were in accordance with literature values [37–41]. Evaluation of ^{13}C-NMR data revealed the predominance of the thione form of compounds containing triazoline and oxadiazoline, hydrazone form of cyclization products and one form of pyrazolone ring.

EI-mass spectra of compounds **7–24** showed molecular ion (M^+) peaks which confirmed their molecular weights. The major fragmentation pathway appeared by the cleavage of –NH–N=C< bonds of the hydrazone moiety [42, 43]. Compounds **7**, **13** and **19** containing a hydrazide-hydrazone structure which were obtained from compound **6a** gave the corresponding fragments by the cleavage of –CO–NH–N=CH– moiety in mass spectrum [21]. EI-mass spectra of compounds **7–24** exhibited the expected fragmentation pattern of hydrazide-hydrazone, triazoline, oxadiazoline and pyrazoline structures.

The triazoline and hydrazone protons of **22** resonated at 14.11 and 13.12 ppm, respectively. In the ^{1}H-NMR spectrum of **22a**, which was obtained from acetylation of **22**, an N–H proton was observed at 13.64 ppm, whilst a signal which is attributable to a triazoline N–H proton was not shown at around 14.00 ppm. This observation also supported that triazoline N–H proton of **22** was acetylated to give **22a** although hydrazone N–H was not affected. Further evidence that monoacetylation occured was obtained by elemental analysis. Another chemical study on compound **22** was carried out to discover whether it could be reduced by refluxing the mentioned compound with hydrazine-hydrate (99%) as reported previously [21, 22]. The identity of the resulting compound **22b**, was confirmed utilizing its melting point and IR spectral data comparing it to those of 5-(4-aminophenyl)-2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-thione as the reference compound, which was reported to be synthesized by a different procedure [12, 44]. It was also observed that **22b** had been co-chromatographed with the above mentioned authentic standard using the TLC system comprising chloroform/methanol (25:75, v/v) as mobile and silicagel 60HF$_{254}$ as stationary phases.

3. Results and discussion

3.1. In vitro antimicrobial activity

The synthesized compounds **7–24** were tested for antibacterial, antifungal and antimycobacterial activities against various strains by the microdilution method [45–49]. The results are given in *table III*. Of the compounds bearing a 1,2,4-triazoline-3-thione ring, **22** was found to be active against *Staphylococcus aureus* ATCC 29213 at a concentration level of 15.6 μg/mL, whereas **9** and **10** had a positive response against the same strain at 31.2 μg/mL. The compounds **7** and **13**, which were obtained from **6a**, were found to be active against the same strain at concentrations of 62.5 and 31.2 μg/mL, respectively. Compound **24** bearing a 1,3,4-oxadiazoline-2(3H)-thione ring exhibited only marginal activity on *S. aureus* ATCC 29213. None of the synthesized compounds had any response against *Escherichia*

Table I. Physical and spectral data for cyclization products **7–24**.

Compound	Molecular formula	M.p. (°C)	Yield (%)	UV ethanol λ_{max} (log ε)	IR (KBr) ν C=O*	EI-MS (m/z, %)
7	$C_{18}H_{15}FN_6O_2$ 366.36	268–269	60	267 (4.07) 408 (4.37)	1 660	366 (M^+) 121 (% 100)
8	$C_{14}H_{15}N_7OS.$ ½ H_2O 338.38	274–275	80	293 (4.54) 395 (4.70)	1 680	329 (M^+, % 100)
9	$C_{15}H_{15}N_7OS.$ ½ H_2O 350.40	263–265	81	204 (4.35) 257 (4.35) 409 (4.37)	1 680	341 (M^+, % 100)
10	$C_{18}H_{15}N_7OS.$ ½ H_2O 386.43	291–294 (dec)	80	204 (4.14) 256 (3.84) 412 (4.02)	1 680	377 (M^+,% 100)
11	$C_{18}H_{15}N_7O_2$ 361.37	294–295	46	259 (4.15) 412 (4.50)	1 690*** 1 680	361 (M^+) 125 (% 100)
13	$C_{20}H_{19}$ F $N_6O_3.$ 2 H_2O 446.41	134	56	293 (4.54) 395 (4.70)	1 660	410 (M^+) 107 (% 100)
14	$C_{16}H_{19}N_7O_2S.$ H_2O 391.44	115	84	258 (4.26) 380 (4.22)	1 660	373 (M^+) 82 (% 100)
15	$C_{17}H_{19}N_7O_2S$ 385.45	67–70	64	259 (4.49) 377 (4.50)	1 665	385 (M^+) 118 (% 100)
17	$C_{20}H_{19}N_7O_3$ 405.42	246–249	45	260 (4.06) 392 (4.44)	1 695*** 1 670	405 (M^+) 259 (% 100)
18	$C_{14}H_{14}N_6O_3$ S. ½ H_2O 355.36	271–272	51	235 (4.06) 306 (4.19) 430 (4.40)	1 660	346 (M^+, % 100)
19	$C_{24}H_{19}F$ N_6O_2 442.45	282–383	61	248 (4.52) 395 (4.68)	1 680	442 (M^+) 77 (% 100)
20	$C_{20}H_{19}N_7OS.$ H_2O 423.49	259–260	55	253 (4.54) 397 (4.52)	1 650	405 (M^+) 77 (% 100)
22	$C_{24}H_{19}N_7OS.$ ½ H_2O 462.53	286–288	48	204 (4.44) 248 (4.26) 404 (4.32)	1 650	453 (M^+) 97 (% 100)
22a	$C_{26}H_{21}N_7O_2S.$ ½ H_2O	220–222	40	248 (4.55) 405 (4.62)	1 740** 1 665	–
23	$C_{24}H_{19}N_7O_2$ 437.46	269–270	49	246 (4.47) 399 (4.54)	1 695*** 1 680	437 (M^+) 77 (% 100)
24	$C_{18}H_{14}N_6O_2S.$ ½ H_2O 387.42	270–273	63	245 (4.40) 300 (4.16)	1 650	378 (M^+) 77 (% 100)

* Pyrazoline C=O, ** acetyl C=O, *** 1,2,4,-triazoline-3(2H)-one C=O. IR spectra of **22b**: 3 400, 3 300 (Ar–NH_2).

coli ATCC 25922 or *Pseudomonas aeruginosa* ATCC 27853. The results also demonstrated that *M. fortuitum* ATCC 6841 was susceptible against **9** with an MIC value of 32 μg/mL. Most of the remaining compounds were found to be active against this pathogen at 64 μg/mL, whilst that of tobramycin, the standard used, was 16 μg/mL. The MIC values of the synthesized compounds **9–12**, **16**, **18** and **21–24** were observed against *Candida albicans* ATCC 2091 at a concentration of 62.5 μg/mL. Only compounds derived from 1,2,4-triazoline-3-thiones or 1,3,4-oxadiazoline-2(3H)-thiones had a positive response against the tested fungal strain. This might indicate the contribution of the acidic protons to the activity.

3.2. Anticonvulsant activity

Some selected compounds were evaluated for anticonvulsant activity against pentylenetetrazole induced seizures in male albino Swiss mice [50, 51]. All the compounds tested at 100 mg/kg showed protection ranging from 10–40% (*table IV*).

3.3. In vitro evaluation of antimycobacterial activity against M. tuberculosis *H37Rv and* M. avium

Compounds **6a–f**, **7–24** and **22a** were also tested for in vitro antituberculosis activity against *M. tuberculosis*

Table II. ^{1}H-NMR spectral data of **7–24**.

Compound	^{1}H-NMR
7 (DMSO-d_6)	2.17 (s, 3H, CH_3); 7.29–7.93 (m, 8H, Ar-H); 9.83 (s, 1H, CH=N); 10.22 (b, 1/2H, =N–NH–CO); 10.54 (b, 1H, pyrazoline N–H); 11.59 (s, 1H, -NH–N=C <)
8 (DMSO-d_6)	1.17 (t, 3H, CH_2–C$\underline{H}_3$); 2.17 (s, 3H, CH_3); 4.05 (q, 2H, C$\underline{H}_2$–CH_3); 7.67–7.71 (m, 4H, Ar-H); 11.59 (s, 1H, pyrazoline N–H); 13.15 (b, 1/2H, -NH–N=C<); 13.88 (s, 1H, triazoline N–H). (pyrazoline, hydrazone and triazoline N–H protons exhanged with deuterium in D_2O)
9 (DMSO-d_6)	2.17 (s, 3H, CH_3); 4.74 (d, 2H, allyl –N–CH_2); 4.89 (d, 1H, allyl =CH, *trans J* = 17 Hz); 5.15 (d, 1H, allyl =CH, *cis J* = 10 Hz); 5.81–5.89 (m, 1H, allyl CH=); 7.68–7.72 (d, 4H, Ar-H); 11.59 (s, 1H, pyrazoline N–H); 13.10 (b, 1/2H, -NH–N=C<); 13.97 (b, 1H, triazoline N–H)
10 (DMSO-d_6)	2.12 (s, 3H, CH_3); 7.32–7.52 (m, 9H, Ar–H); 11.56 (s, 1H, pyrazoline N–H); 14.06 (b, 1H, triazoline N–H)
11 (DMSO-d_6)	2.12 (s, 3H, CH_3); 7.26–7.98 (m, 9H, Ar-H); 11.49 (s, 1H, pyrazoline N–H); 12.08 (s, 1H, triazoline N–H); 13.00 (b, 1/2H, -NH–N=C<)
13 (DMSO-d_6)	2.21 (s, 3H, CH_3); 2.50 (m, 2H, -N–CH_2); 3.64–3.71 (m, 2H, -CH_2–C$\underline{H}_2$–OH); 4.78 (s, 1H, -CH_2–CH_2–O$\underline{H}$); 7.30–7.99 (m, 8H, Ar–H); 8.47 (s, 1H, CH=N); 11.49 (b, 1/2H, =N–NH–CO); 11.82 (s, 1H, -NH–N=C<)
14 (DMSO-d_6 + $CDCl_3$)	1.18 (t, 3H, CH_2–C$\underline{H}_3$); 2.19 (s, 3H, CH_3); 2.50 (m, 2H, -N–CH_2); 3.64–3.71 (m, 2H, -CH_2–C$\underline{H}_2$–OH); 4.07 (q, 2H, C$\underline{H}_2$–CH_3); 4.78 (s, 1H, -CH_2–CH_2–O$\underline{H}$); 7.46–7.80 (m, 4H, Ar–H); 11.59 and 11.85 (2s, 1H, -NH–N=C<); 13.84 (b, 1H, triazoline N–H)
15 (DMSO-d_6)	2.19 (s, 3H, CH_3); 2.50 (m, 2H, -N–CH_2); 3.64–3.71 (m, 2H, -CH_2–C$\underline{H}_2$–OH); 4.75 (m, 3H, allyl –N–CH_2 and -CH_2–CH_2–O$\underline{H}$); 4.89 (d, 1H, allyl =CH, *trans J* = 17 Hz); 5.15 (d, 1H, allyl =CH, *cis J* = 10 Hz); 5.85 (m, 1H, allyl CH=); 7.69–7.73 (m, 4H, Ar–H); 11.30 and 11.59 (2s, 1H, -NH–N=C<); 13.98 (b, 1H, triazoline N–H)
17 (DMSO-d_6)	2.19 (s, 3H, CH_3); 2.50 (m, 2H, -N–CH_2); 3.57–3.65 (m, 2H, -CH_2–C$\underline{H}_2$–OH); 4.77 (s, 1H, -CH_2–CH_2–O$\underline{H}$); 7.23–7.91 (m, 9H, Ar–H); 12.51 (b, 1H, triazoline N–H); 13.11 (s, 1H, -NH–N=C <)
18 (DMSO-d_6)	2.19 (s, 3H, CH_3); 2.50 (m, 2H, -N–CH_2); 3.57–3.65 (m, 2H, -CH_2–C$\underline{H}_2$–OH); 4.76 (s, 1H, -CH_2–CH_2–O$\underline{H}$); 7.71–7.91 (m, 4H, Ar–H); 13.17 (s, 1H, -NH–N=C<); 14.69 (b, 1H, oxadiazoline N–H)
19 (DMSO-d_6)	2.33 (s, 3H, CH_3); 7.24–7.96 (m, 13H, Ar-H); 9.85 (s, 1H, CH=N); 10.26 (s, 1H, =N–NH–CO); 12.77 (s, 1H, -NH–N=C<)
20 (DMSO-d_6)	1.18 (t, 3H, CH_2–C$\underline{H}_3$); 2.32 (s, 3H, CH_3); 4.08 (q, 2H, C$\underline{H}_2$–CH_3); 7.23–7.92 (m, 9H, Ar-H); 13.90 (s, 1H, triazoline N–H)
22 (DMSO-d_6)	2.28 (s, 3H, CH_3); 7.22–7.89 (m, 14H, Ar–H); 13.12 (b, 1/2H, -NH–N=C<); 14.11 (b,1H, triazoline N–H)
22a (DMSO-d_6)	2.27 (s, 3H, CH_3); 2.75 (s, 3H, -$COCH_3$); 7.22–7.89 (m, 14H, Ar–H); 13.64 (b, 1/2H, -NH–N=C <)
23 (DMSO-d_6)	2.32 (s, 3H, CH_3); 7.23–8.01 (m, 14H, Ar-H); 12.56–13.63 (b, 2H, triazoline and -NH-N=C <).
24 (DMSO-d_6)	2.30 (s, 3H, CH_3); 7.23–7.91 (m, 9H, Ar–H); 13.24 (b, 1H, oxadiazoline N–H).

H37Rv using the BACTEC 460 radiometric system [52, 53]. Rifampicin was used as the standard in the tests. Primary antituberculosis activity screening results of these compounds can be seen in *table V*. The compounds, which exhibited < 90% inhibition in the primary screen (MIC > 12.5 μg/mL) were not evaluated further. Compound **24** effecting > 90% inhibition in the primary screen at 12.5 μg/mL were re-tested at lower concentrations against *M. tuberculosis* H37Rv to determine the actual minimum inhibitory concentration in a broth microdilution assay using Alamar Blue. The remaining compounds were found to be inactive in the level I assay. The MIC was defined as the lowest concentration inhibiting 99% of the inoculum. A level II assay revealed that the MIC value was not less than 12.5 μg/mL. Compound **24** was also tested against *M. avium*, a naturally drug-resistant opportunistic pathogen, using the same technique. Clarithromycin was used as the standard in the assays. However, no inhibition was observed against *M. avium* with compound **24** at 12.5 μg/mL, whereas clarithromycin exhibited 98% inhibition at 2 μg/mL. Level II assay results of **24** are given in *table V*.

Although all test compounds were dissolved in DMSO, it was observed that the solvent showed no activity in these assays at the level that was used for screening. This was most probably because compounds were initially dissolved in DMSO in the minimal volume, then serially diluted with media to the testing concentration, so there was very minimal residual DMSO in the assayed medium. Controls were run in each assay to verify this fact.

4. Experimental protocols

4.1. Chemistry

Ethyl isothiocyanate, phenyl isothiocyanate and 4-fluorobenzaldehyde, β-hydroxy-ethylhydrazine and hydrazine hydrate were purchased from Sigma and Fluka. All other chemicals were purchased from Merck.

Table III. Microbiological results of 4-arylhydrazono-2-pyrazoline-5-ones **7–24**.

Compound	S. aureus ATCC 29213	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*M. fortuitum* ATCC 6841	*C. albicans* ATCC 2091
7	62.5	250	125	64	125
8	250	250	125	64	125
9	31.2	250	125	32	62.5
10	31.2	250	125	64	62.5
11	250	250	125	64	62.5
12	250	250	125	64	62.5
13	31.2	250	125	64	500
14	250	250	125	64	125
15	250	250	250	64	125
16	250	250	125	64	62.5
17	250	250	125	64	62.5
18	250	250	125	64	62.5
19	500	250	125	64	125
20	500	250	125	> 128	125
21	500	250	125	128	62.5
22	15.6	125	125	64	62.5
23	250	250	125	64	500
24	62.5	250	125	64	62.5
Ceftriaxone	4	0.03	8	–	–
Tobramycin	–	1	2	16	–
Miconazole	–	–	–	–	0.05

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- and ^{13}C-) were recorded on a Bruker AVANC-DPX 400 spectrometer. Mass spectra were obtained on a Fisons Instruments VG Platform II LC-MS in the electron impact mode. TLC was carried out on pre-coated plates (Silicagel 60 HF254

Table IV. Anticonvulsant screening test results.

Compound	Protection (%)
4d	10
5d	10
6d	10
6e	10
6f	20
10	30
11	40
12	10
16	10
17	20
18	30
22	20
23	10
24	10

Table V. Antimycobacterial activity of Level I assays of **6a–f**, **7–12** and **14–24**.

Compound	MIC (mg/mL)	% Inhibition	Level II assay
6a	> 12.5	62	**–**
6b	> 12.5	0	–
6c	> 12.5	0	–
6d	> 12.5	0	–
6e	> 12.5	0	–
6f	> 12.5	13	–
7	> 12.5	5	–
8	> 12.5	0	–
9	> 12.5	0	–
10	> 12.5	0	–
11	> 12.5	0	–
12	> 12.5	0	–
14	> 12.5	0	–
15	> 12.5	4	–
16	> 12.5	9	–
17	> 12.5	0	–
18	> 12.5	0	–
19	> 12.5	25	–
20	> 12.5	73	–
21	> 12.5	40	–
22	> 12.5	17	–
22a	> 12.5	51	–
23	> 12.5	0	–
24	< 12.5	91	>12.5

Merck Art. 105554) employing the solvent systems petroleum ether:acetone (50:50, v/v) and benzene:methanol (70:30, v/v).

4.1.1. Preparation of aromatic primary amines

Compounds containing aromatic primary amine function were prepared as described previously [12, 18–20].

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

The coupling products were synthesized by the reactions of diazonium salts of aromatic primary amines with ethylacetotacetate [18].

4.1.3. Synthesis of 4-arylhydrazono-2-pyrazoline-5-ones **7–24**

The coupling products **6a–f** (0.01 mol) were refluxed with substituted hydrazines (0.01 mol) in glacial acetic acid to give **7–24**. Physical, analytical and spectral data for these cyclization products were given in *tables I* and *II*.

4.1.3.1. 3-Methyl-4[4-[(1,3,4-oxadiazol-2(3H)-thione-5-yl)phenyl]hydrazono]-2-pyrazoline-5-one **12**

$C_{12}H_{10}N_6O_2S.1/2H_2O$. Yield: 61%, m.p.: 285 °C. UV (EtOH) λ_{max} (log ε) = 432 (4.32) nm, 305 (4.09) nm, IR (KBr) = 3 300–3 150, 2 900, 1 680, 1 610, 1 550, 1 500, 1 420, 1 390, 1 340, 1 240, 1 180, 870, 840. ^{1}H-NMR (400 MHz, DMSO-d_6 + $CDCl_3$) ppm = 2.22 (s, 3H, pyrazoline C$\underline{H}_3$), 7.61 (d, 2H, o-NH, J = 8.6 Hz), 7.93 (d, 2H, m-NH, J = 8.6 Hz), 11.45 (s, 1H, pyrazoline N–H). ^{13}C-NMR (100.6 MHz, DMSO-d_6) ppm: 12.42 (pyrazoline $\underline{C}H_3$), 116.81 (C2 and C6), 118.98 (C4), 128.37 (C3 and C5), 130.70 (C1), 145.21 (hydrazone, C=N), 147.81 (pyrazoline, C=N), 160.78 (oxadiazoline, C=N), 161.02 (pyrazoline, C=O), 178.12 (oxadiazoline, C=S). EI-MS (70 eV, m/z): 304, 303, 302 (M^+, % 100), 242, 227, 194, 193, 192, 133, 132, 126, 125, 119, 118, 98, 97, 92, 85, 84.

4.1.3.2. 1-(β-Hydroxyethyl)-3-methyl-4[4-[(2,4-dihydro-4-phenyl-3H-1,2,4-triazole-3-thione-5-yl)phenyl]hydrazono]-2-pyrazoline-5-one **16**

$C_{20}H_{19}N_7O_2S$. Yield: 79%, m.p.: 265–268 °C. UV (EtOH) λ_{max} (log ε) = 400 (4.36) nm, 253 (4.15) nm, 205 (4.47) nm. IR (KBr) = 3 500–3 400, 3 250, 3 100, 2 900, 1 680, 1 615, 1 590, 1 560, 1 420, 1 380, 1 340, 1 250, 1 180, 860, 840, 700. ^{1}H-NMR (400 MHz, DMSO-d_6 + $CDCl_3$) ppm = 2.22 (s, 3H, pyrazoline C$\underline{H}_3$), 2.58 (t, 2H, N–C$\underline{H}_2$), 3.51 (m, 2H, CH_2–C$\underline{H}_2$–OH), 3.88 (s, 1H, -CH_2–CH_2–O$\underline{H}$), 7.27–7.52 (m, 9H, Ar-H). ^{13}C-NMR (100.6 MHz, DMSO-d_6) ppm: 12.27 (pyrazoline $\underline{C}H_3$), 47.03 (-N–$\underline{C}H_2$–CH_2–OH), 59.09 (-N–CH_2–$\underline{C}H_2$–OH), 116.44 (C2 and C6), 122.30 (C4), 122.71 (C8 and C12), 125.60 (C10), 129.60 (C1), 130.27 (C9 and C11), 130.36 (C3 and C5), 135.47 (C7), 143.77 (hydrazone, C=N), 147.05 (pyrazoline, C=N), 150.95 (triazoline, C=N), 158.37 (pyrazoline, C=O), 169.49 (triazoline, C=S). EI-MS (70 eV, m/z): 422, 421 (M^+), 269, 268, 267, 253, 252, 192, 169, 151, 137, 133, 132, 119, 118, 117 (% 100), 109, 92, 77.

4.1.3.3. 3-Methyl-1-phenyl-4[4-[(4-allyl-2,4-dihydro--3H-1,2,4-triazole-3-thione-5-yl)phenyl]hydrazono]-2-pyrazoline-5-one **21**

$C_{21}H_{19}N_7OS.1/2H_2O$. Yield: 78%, m.p.: 230–234 °C. UV (EtOH) λ_{max} (log ε) = 400 (4.31) nm, 253 (4.30) nm. IR (KBr) = 3 250, 3 150, 2 900, 1 670, 1 610, 1 590, 1 550, 1 490, 1 420, 1 380, 1 340, 1 260, 1 180, 700, 690. ^{1}H-NMR (400 MHz, DMSO-d_6 + $CDCl_3$) ppm = 2.35 (s, 3H, pyrazoline C$\underline{H}_3$), 4.71 (d, 2H, allyl -N–C$\underline{H}_2$), 5.01 (d, 1H, allyl =C$\underline{H}$, *trans*, J = 17 Hz), 5.23 (d, 1H, allyl =C$\underline{H}$, *cis*, J = 9 Hz), 5.89–5.96 (m, 1H, allyl C$\underline{H}$=), 7.20–7.94 (m, 9H, Ar–H), 13.77 (b, 1/2H, triazoline N–H). ^{13}C-NMR (100.6 MHz, DMSO-d_6) ppm: 12.45 (pyrazoline $\underline{C}H_3$), 46.88 (-N–$\underline{C}H_2$), 117.24 (allyl, =$\underline{C}H_2$), 118.16 (C2 and C6), 118.57 (C8 and C12), 123.35 (C4), 125.75 (C10), 129.85 (C9 and C11), 129.91 (C1), 130.46 (C3 and C5), 132.71 (allyl, -$\underline{C}$H=CH_2), 138.66 (C7), 144.07 (hydrazone, C=N), 149.52 (pyrazoline, C=N), 151.67 (triazoline, C=N), 157.16 (pyrazoline, C=O), 168.51 (triazoline, C=S). EI-MS (70 eV, m/z): 419, 418, 417 (M^+), 402, 389, 385, 384, 233, 232, 231, 217, 216, 202, 201, 192, 187, 186, 174, 173, 159, 144, 133, 132, 119, 118, 99, 92.

4.2. Microbiology and pharmacology

4.2.1. 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 micro-organism 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 McFarland 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 compound that inhibited micro-organism growth as detected by the unaided eye, was recorded to represent the MIC expressed in μg/mL.

4.2.2. Antimycobacterial activity

Preparation of mycobacterial inocula 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 McFarland 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. Tobramycin, 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*. Tobramycin 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, containing 100 μL of broth containing tobramycin or the test compounds, were then inoculated with 10 μL of *M. fortuitum* suspension whose preparation is 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.

4.2.3. In vitro evaluation of antimycobacterial activity against M. tuberculosis H37Rv and M. avium

A primary screen was conducted at 12.5 μg/mL (or molar equivalent of highest molecular weight compound in a series of congeners) against *M. tuberculosis* H37Rv in BACTEC 12B medium using the BACTEC 460 radiometric system [52, 53]. Compounds effecting < 90% inhibition in the primary screen (MIC > 12.5 μg/mL) were not evaluated further. Compounds demonstrating at least 90% inhibition in the primary screen were re-tested at lower concentration (MIC) in a broth microdilution assay with Alamar Blue. The MIC was defined as the lowest concentration inhibiting 99% of the inoculum. These compounds were also tested against *M. avium*, a naturally drug-resistant opportunistic pathogen, using the same technique. Concurrent with the determination of MICs, compounds were tested for cytotoxicity (IC_{50}) in VERO cells at concentrations equal to and greater than the MIC for *M. tuberculosis* H37Rv. After 72 h exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 Non-radioactive Cell Proliferation Assay.

4.2.4. BACTEC radiometric method of susceptibility testing

Inocula for susceptibility testing were either from a positive BACTEC isolation vial with a growth index (GI) of 500 or more, or a suspension of organisms isolated earlier on a conventional medium.

The culture was well mixed with a syringe and 0.1 mL of a positive BACTEC culture was added to each of the vials containing the test drugs. The drug vials contained rifampicin (0.25 μg/mL). A control vial was inoculated with a 1:100 dilution of the culture. A suspension equivalent to a McFarland No. 1 standard was prepared in the same manner as a BACTEC positive vial, when growth from a solid medium was used.

Each vial was tested immediately on a BACTEC instrument to provide CO_2 in the headspace. The vials were incubated at 37 °C and tested daily with a BACTEC instrument. When the GI in the control read at least 30, the increase in GI (Δ GI) from the previous day in the control was compared with that in the drug vial. The following formula was used to interpret results:

Δ GI control > Δ GI drug = susceptible

Δ GI control < Δ GI drug = resistant

If a clear susceptibility pattern (the difference of Δ GI of control and the drug bottle) was not seen at the time the control GI was 30 the vials were read for 1 or 2 additional days to establish a definite pattern of Δ GI differences.

4.2.5. Anticonvulsant activity

The anticonvulsant activity of some compounds was determined against pentylenetetrazole-induced seizures in albino Swiss mice [50, 51] of either sex weighing 20–30 g. They were housed in groups of 15 and acclimatized to their environment for at least 2 days before the experiments were done. The animals were allowed free access to food and water before being tested. The test compounds were suspended in 5% aqueous suspension of gum acacia. These compounds were administered to a group of 10 animals at a dose of 100 mg/kg intraperitoneally. Two hours after the administration mice were injected with pentylenetetrazole (90 mg/kg) subcutaneously. This dose of pentylenetetrazole has been shown not only to produce convulsions in almost all untreated mice, but also to exhibit 100% mortality during the 24 h period in the control group. The mice were observed for the next 60 min for the occurrence of seizures. Animals devoid of a threshold convulsion were considered protected. The mortality within 24 h was also recorded.

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