Synthesis, Spectral Characterization, Cytotoxic, and Antimicrobial Activities of Some Novel Heterocycles Utilizing 1,3-Diphenylpyrazole-4-carboxaldehyde Thiosemicarbazone

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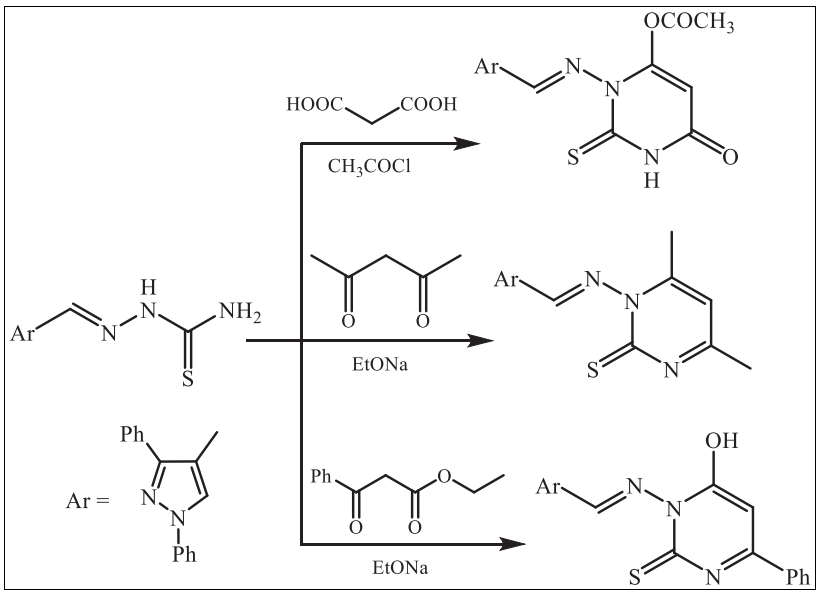


A new series of heterocycles was synthesized *via* the reaction of readily obtainable 1,3-diphenylpyrazole-4-carboxaldehyde thiosemicarbazone with many carbon electrophiles, for example, chloroacetic acid, chloroacetyl chloride, ethyl chloroacetate, dimethyl acetylenedicarboxylate, maleic anhydride, 3′-nitro-ω-bromoacetophenone, malonic acid, acetylacetone, ethyl benzoylacetate, arylidene malononitrile, and ethyl cyanoacetate in attempt to construct imidazolidinone, thiazolidinone, thiazole, and pyrimidine derivatives. The behavior of the titled compound towards hydrazine hydrate was investigated, in addition to the ring closure under different conditions. Also, the reactions with 2-chloroquinoline-3-carboxaldehyde and chromone-3-carboxaldehyde were discussed. The structures of all products obtained were substantiated from their analytical and spectral data. The antitumor and antimicrobial activities of the synthesized compounds were examined.

INTRODUCTION

Pyrazole derivatives occupy a unique place in the medicinal chemistry due to their extensive applications, for example, antitumor [1–4], antioxidant [5], anti-inflammatory [6], antimicrobial [7–10], and antiviral activities [11–14]. Also, it has been documented that newly synthesized pyrazole derivatives known as potential anticancer agent due to their B-Raf inhibitory activities with IC_{50} values in the nano levels, for example, compounds **I–IV** (Fig. 1) [15,16]. 1,3-Diphenylpyrazole-4-carboxaldehyde thiosemicarbazone was utilized to construct several *N*-heterocycles [17]. Accordingly, it seemed interesting to carry out the transformation of the titled compound into many *N*-heterocycles bearing pyrazole moiety as a platform to evaluate them as antitumor and antimicrobial agents.

RESULTS AND DISCUSSION

Chemistry. 1,3-Diphenylpyrazole-4-carboxaldehyde thiosemicarbazone **2** was easily synthesized from the condensation of the titled aldehyde **1** with thiosemicarbazide [17]. The reaction of thiosemicarbazone **2** with chloroacetic acid was discussed under different conditions. Therefore, when the reaction was carried out in refluxing pyridine for 4 h, the thioxoimidazolidinone derivative **3** was obtained in 74% yield. While the reaction in refluxing ethanol containing anhydrous sodium acetate led to the construction of thiazolidinone **4** in 79% yield (Scheme 1). The structures of these products were inferred from their analytical and spectral data. The infrared (IR) spectrum of **3** revealed the characteristic absorption band for the imidazolidinone C═O at ν1718 cm^{-1} as well as C═S at ν1248 cm^{-1}. The ^{1}H-NMR spectrum displayed a

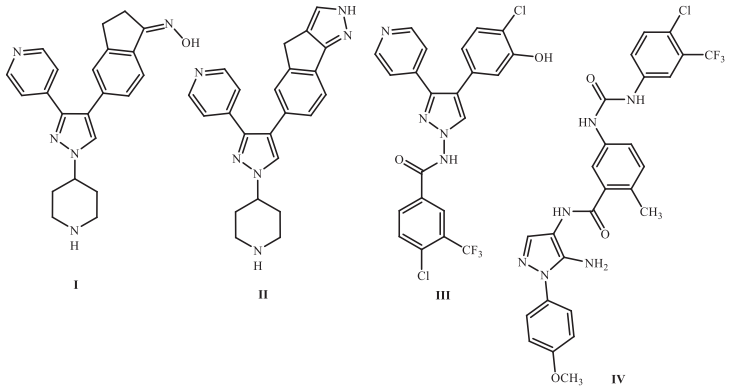
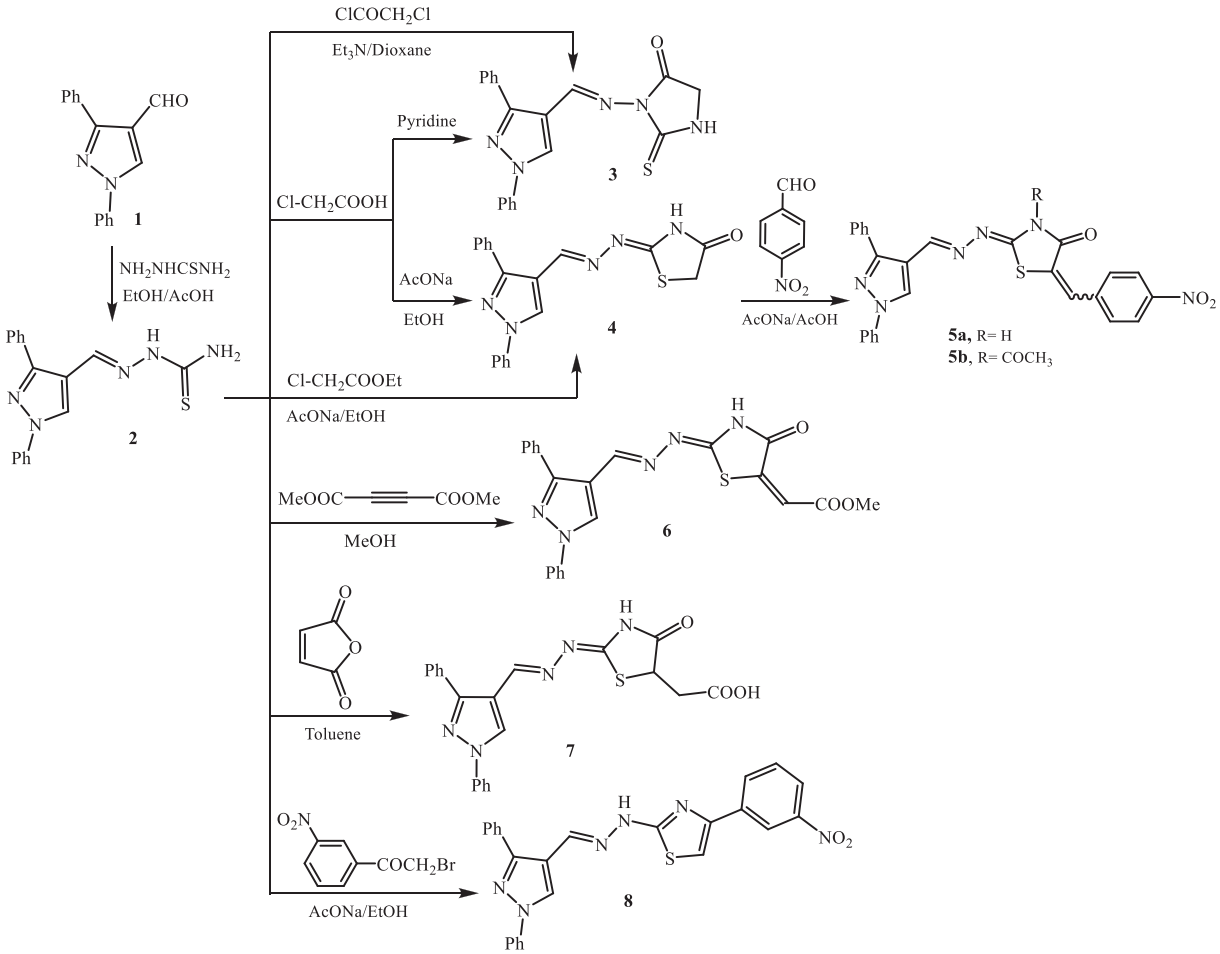


Figure 1. Structures of some potent pyrazole derivatives.

Scheme 1. Synthetic pathways for the formation of compounds **3**–**8**.



singlet signal at δ 11.86 ppm integrated to one proton exchangeable with D_2O, which is attributed to NH of imidazolidine moiety, in addition to a singlet at δ 3.89 ppm integrated to two protons due to CH_2 protons. The IR spectrum of **4** was devoid from *v*C═S and showed the characteristic absorption band for the thiazolidinone C═O at *v*1717 cm^{-1}. The ^{1}H-NMR provided a singlet at δ 12.05 ppm integrated to one proton exchangeable with D_2O attributable for NH of thiazolidine moiety, in addition to a singlet at δ 3.95 ppm due to CH_2 protons. Interestingly, compounds **3** and **4** can also be prepared in fairly good yields through alternative routes *via* the

reaction of thiosemicarbazone **2** with chloroacetyl chloride in the presence of triethylamine/dioxane and ethyl chloroacetate in the presence of anhydrous sodium acetate/ethanol, respectively, which can be proved by melting point, mixed melting point, thin layer chromatography (TLC), and IR.

The aldol condensation of the methylene group at position-5 of thiazolidinone **4** with *p*-nitrobenzaldehyde in refluxing AcONa/AcOH afforded *α,β*-unsaturated carbonyl compounds **5a**,**b**. The formation of **5b** could be interpreted upon acetylation *via* the reaction medium used. The structures of compounds **5a**,**b** were elucidated from their analytical and spectral data. The IR spectrum of compound **5a** displayed a broad absorption band 3432 cm^{-1} (*v*OH) indicating to its existence as a mixture of lactam and lactim forms. The IR spectrum of **5b** was devoid from *v*NH and provided the characteristic absorption bands of C═O groups at *v*1715 and 1673 cm^{-1} due to mechanical coupling between the two carbonyl groups of imide linkage. The ^{1}H-NMR spectrum of **5b** was lacked to any labile hydrogen and showed its existence as a mixture of *E*-isomer and *Z*-isomer in a ratio of 80:20%, respectively (cf. Experimental). The presence of methyl group in compound **5b** was approved by the appearance of singlet signal in its ^{1}H-NMR spectrum at δ 1.91 ppm integrated to three protons.

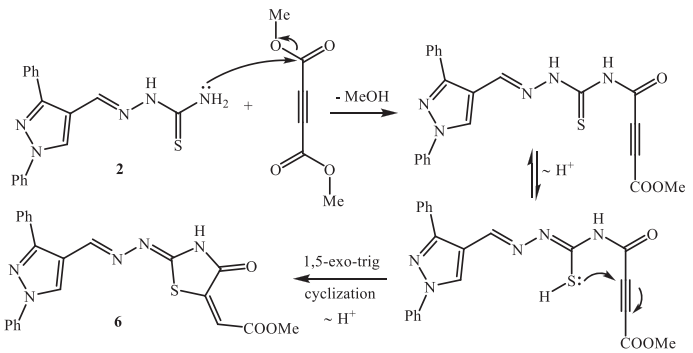
Dimethyl acetylenedicarboxylate condensed with thiosemicarbazone **2** in refluxing methanol for 1 h to furnish thiazolidinone **6** in 82% yield (Scheme 1). The IR spectrum of compound **6** displayed the C═O of α,β-unsaturated ester group at *v*1713 cm^{-1} in addition to C═O of thiazolidinone moiety at *v*1700 cm^{-1}. The ^{1}H-NMR spectrum showed singlet for NH proton at δ 12.78 ppm (exchangeable with D_2O) in addition to methyl protons singlet at δ 3.81 ppm. The reaction could be visualized to occur *via* the nucleophilic attack of the terminal NH_2 on the ester carbonyl group to eliminate methanol molecule followed by ring closure (Scheme 2).

A facile synthesis of 4-oxothiazolidin-5-ylacetic acid **7** was provided by treatment of **2** with maleic anhydride in refluxing toluene for 5 h [18]. On the other hand, refluxing **2** with 3′-nitro-ω-bromoacetophenone in ethanolic solution containing anhydrous sodium acetate brings about the formation of thiazole derivative **8**. The structure of **8** was substantiated from its analytical and spectral data. The IR spectrum was lacked to *v*C═O and showed *v*NH at 3396 cm^{-1}. The ^{1}H-NMR spectrum was completely compatible with the assigned structure. All the foregoing reactions are illustrated by Scheme 1.

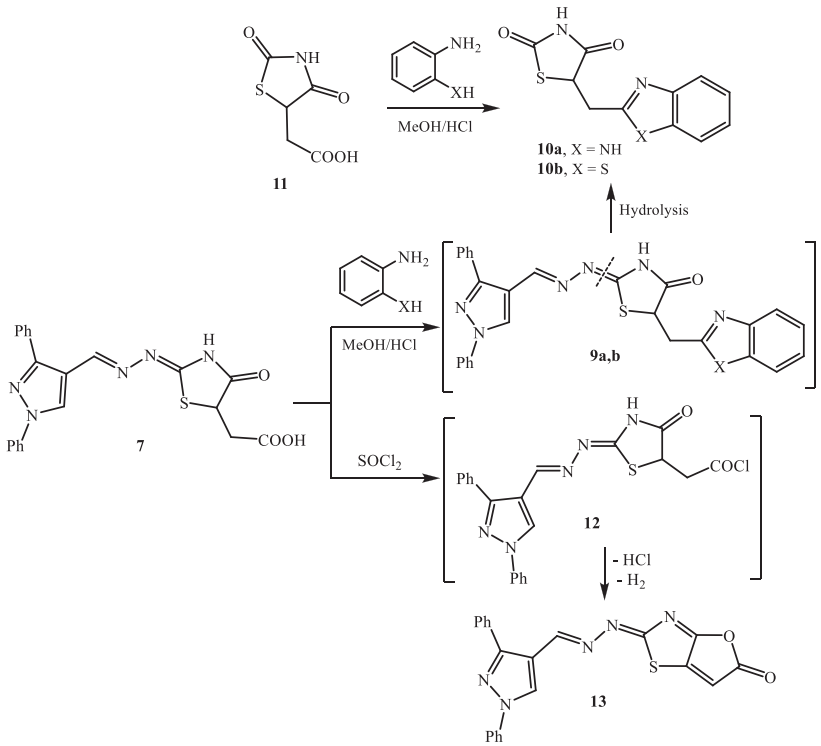
The behavior of 4-oxothiazolidin-5-ylacetic acid **7** towards bis-nucleophiles was investigated. Thus, its condensation with *o*-phenylenediamine and/or *o*-aminothiophenol in refluxing methanol containing catalytic amount of HCl afforded benzimidazole **10a** and/or benzothiazole **10b** derivatives, respectively. The structures of **10a**,**b** were confirmed from the study of the IR together with chemical evidence by direct comparison with authentic samples prepared from the reaction of the 2,4-dioxothiazolidin-5-ylacetic acid **11** with *o*-phenylenediamine and/or *o*-aminothiophenol in refluxing methanol/HCl [19] (identity: melting point, mixed melting point, TLC). The formation of the products **10a**,**b** could be proceed *via* the elimination of two water molecules to form the nonisolable intermediate **9a**,**b** followed by hydrolysis as depicted in Scheme 3. The reaction of acid **7** with thionyl chloride led to the formation of furothiazole derivative **13** *via* the formation of the intermediate acid chloride **12** followed by the elimination of HCl and H_2 molecules (Scheme 3). The structure of **13** was established from its analytical and spectral data. The IR spectrum displayed *v*C═O at 1734 cm^{-1} attributable for *α,β*-unsaturated C═O group of furanone moiety, as well as the absence of any labile hydrogens in its ^{1}H-NMR spectrum (cf. Experimental).

The heterocyclic ring closure of thiosemicarbazone **2** under two different reaction conditions was studied. Therefore, refluxing **2** in pyridine for 6 h led to the construction of triazolethione **14**. While refluxing in an equimolar mixture of HCl/CH_3COOH for 2 h afforded the fused heterocyclic compound, namely,

Scheme 2. A plausible mechanistic pathway for the formation of thiazolidinone **6**.

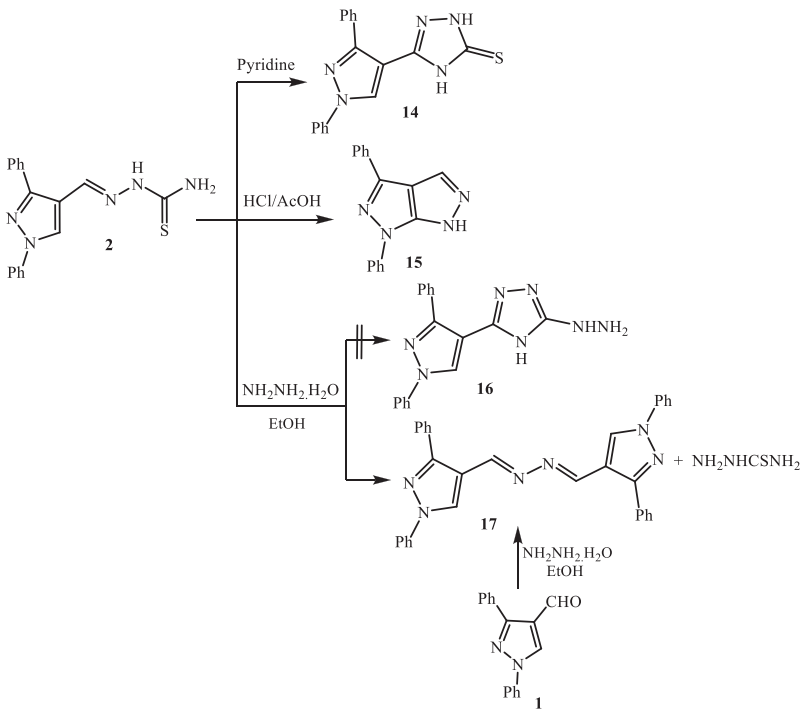


Scheme 3. Synthetic pathways for compounds **10a**,**b** and **13**.



1,3-diphenyl-1,6-dihydropyrazolo[3,4-*c*]pyrazole **15** (Scheme 4). The structures of these compounds were substantiated from their analytical and spectral data. The ^{1}H-NMR spectrum of **14** indicated to its existence as a mixture of thiolactam ⇌ thiolactim isomers in a ratio of 1:1. It displayed two NH protons at δ 9.30 and 8.55 ppm,

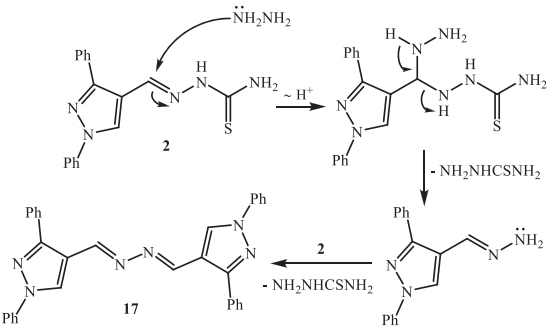
Scheme 4. Synthetic pathways for the formation of compounds **14**, **15**, and **17**.



as well as SH proton at δ 8.85 ppm. The ^{1}H-NMR spectrum of **15** exhibited only one labile hydrogen at δ 13.20 ppm attributable for NH of pyrazole moiety. The formation of pyrazolopyrazole **15** could be explained *via* 1,5-endo-trig cyclization by nucleophilic attack of the NH on C-5 of pyrazole moiety followed by elimination of H—N=C=S molecule.

Hydrazinolysis of **2** failed to produce the hydrazinotriazole **16** and furnished a mixture of the diheteryl azine **17** and thiosemicarbazide (cf. Scheme 4). The structure of **17** was confirmed from the study of the IR together with chemical evidence by direct comparison with an authentic sample prepared from reaction of 1,3-diphenylpyrazole-4-carboxaldehyde **1** with hydrazine hydrate in refluxing ethanol [8] (identity melting point, mixed melting point, TLC). Presumably, the formation of azine **17** could be achieved *via* Scheme 5.

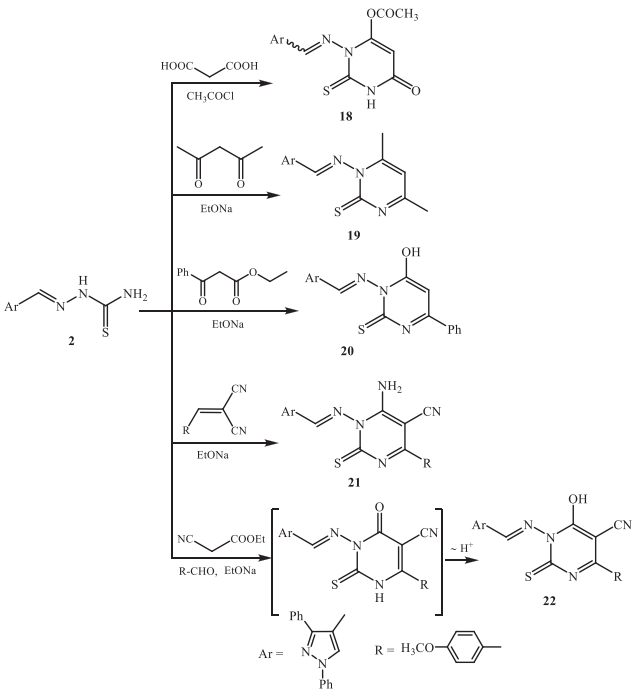
Scheme 5. A suggested mechanistic pathway for the formation of azine **17**.



This work extended to utilize thiosemicarbazone **2** to construct pyrimidine heterocycles. Indeed, treatment of **2** with malonic acid in 5 ml of acetyl chloride afforded the pyrimidinethione **18** (Scheme 6). The structure of **18** was elucidated from its analytical and spectral data. The IR spectrum provided the characteristic absorption bands of C=O at *ν*1721 and 1672 cm^{-1} attributable for ester and amide groups, respectively, as well as NH group at *ν*3257 cm^{-1} and C=S group at *ν*1213 cm^{-1}. The ^{1}H-NMR spectrum showed its existence as a mixture of *E*-isomer and *Z*-isomer in a ratio of 1:1. Thus, it displayed two singlets at δ 2.03 and 1.97 ppm each integrated to three protons attributable for CH_3 protons, as well as two singlets for NH proton at δ 10.84 and 10.79 ppm exchangeable with D_2O (cf. Experimental).

Refluxing **2** with either acetylacetone, ethyl benzoylacetate or 2-(4-methoxybenzylidene) malononitrile in refluxing sodium ethoxide solution led to the formation of pyrimidine derivatives **19–21**. The structures of compounds **19–21** were demonstrated from their analytical and spectral data. The IR spectra were devoid from the characteristic absorption bands of *ν*C=O group. The ^{1}H-NMR spectrum of **19** displayed two singlets at δ 2.25 and 2.22 ppm each integrated to three protons attributable for two methyl protons. The ^{1}H-NMR spectrum of **21** exhibited broad singlet at δ 6.50 ppm integrated to two protons exchangeable with D_2O attributable for NH_2 protons. Interestingly, 6-hydroxy-5-

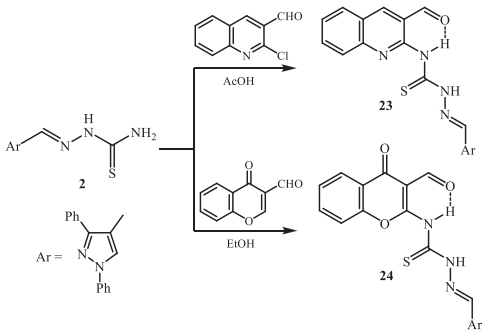
Scheme 6. Synthetic pathways for the formation of compounds **18–24**.



cyanopyrimidine **22** was synthesized *via* a one-pot cyclocondensation reaction between equivalent molar quantities of thiosemicarbazone **2**, ethyl cyanoacetate, and *p*-anisaldehyde in refluxing sodium ethoxide solution (Scheme 6). The structure of **22** was deduced from its analytical and spectral data. The IR spectrum revealed the absence of the characteristic absorption bands of *v*C=O group and the appearance of *v*OH as well as *v*C≡N groups at 3445 cm^{-1} and 2215 cm^{-1}, respectively. The ^{1}H-NMR spectrum displayed a singlet for −OH proton at δ 11.31 ppm exchangeable with D_2O, as well as a singlet at δ 3.79 ppm integrated to three protons attributable for $-OCH_3$ protons (cf. Experimental).

On the other hand, reaction of 2-chloroquinoline-3-carboxaldehyde with thiosemicarbazone **2** in refluxing glacial acetic acid for 6 h afforded quinoline derivative **23**. The IR spectrum exhibited broad absorption band at *v*3415 cm^{-1} attributable for H-bonded NH, as well as C=O at *v*1662 cm^{-1}. The ^{1}H-NMR spectrum exhibited two singlets at δ 11.30 and 10.50 ppm each integrated to one proton attributable for two NH protons. The formation of **23** could be proceed *via* nucleophilic attack of the terminal NH_2 group in thiosemicarbazone **2** on position-2 of quinoline nucleus to eliminate HCl molecule. Also, the reaction of chromone-3-carboxaldehyde with **2** in refluxing ethanol underwent Michael addition on *C-2* of the chromonic moiety followed by dehydrogenation to produce chromone derivative **24** (Scheme 7). The structure of **24** was substantiated from its analytical and spectral data. The IR spectrum of **24** showed a broad band at *v*3427 cm^{-1} attributable for hydrogen bonded NH. The ^{1}H-NMR spectrum was completely consistent with the assigned structure (cf. Experimental).

Scheme 7. Synthetic pathways for the formation of compounds **23** and **24**.



Biological activity. ***Antimicrobial activity screening.*** The reported biological activities of most of the hetero-ring systems synthesized in the present investigation promoted our interest to evaluate the antibacterial and antifungal activities of synthesized compounds using the diffusion disc method [20].

The experiments were performed using test bacterial organisms belonging to the Gram-positive and Gram-negative, namely, *Staphylococcus aureus* and *Escherichia coli*, respectively, as well as *Aspergillus flavus* and *Candida albicans* as tested fungi; Ampicillin was used as standard drug for bacteria, and amphotericin B was used as standard drug for fungi. Preliminary screening of the synthesized compounds and standard drugs was performed at fixed concentration 20 mg/mL. Inhibition was recorded by measuring the diameter of the inhibition

Table 1

Antibacterial and antifungal activity (as inhibition zone in mm diameter).

	Antibacterial activity		Antifungal activity	
Sample	*Escherichia coli* (G^-)	*Staphylococcus aureus* (G^+)	*Aspergillus flavus*	*Candida albicans*
3	12	14	11	0
4	13	12	5	0
6	12	11	5	0
7	23	20	15	18
8	8	5	7	0
13	7	5	0	0
14	12	13	11	10
15	10	10	13	11
18	12	12	11	10
19	0	2	3	0
20	11	12	10	8
21	12	13	14	10
22	14	13	15	12
23	16	15	13	10
24	18	19	15	11
Ampicillin	25	21	—	—
Amphotericin B	—	—	16	21

G: Gram reaction 0.0: no activity (inhibition zone less than 7 mm). 7–10: Weak activity 11–15: moderate activity. More than 15: strong activity.

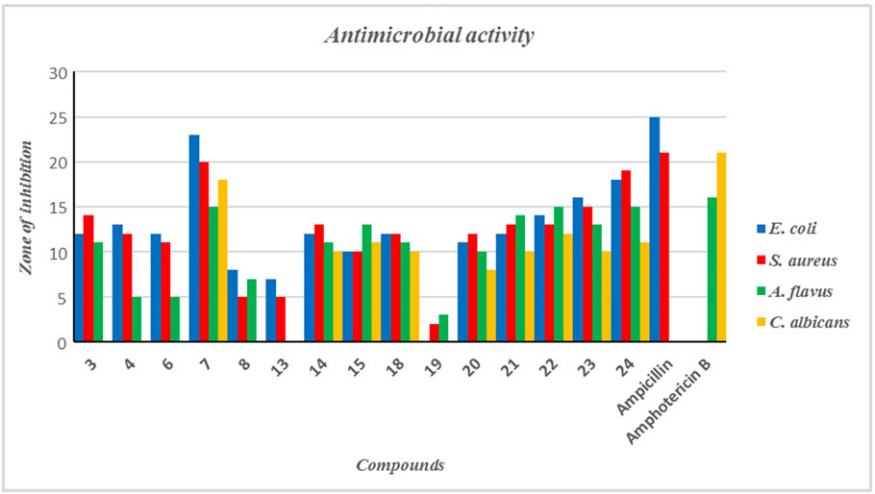


Figure 2. The effect of compounds on inhibition zone diameters against the tested organisms. [Color figure can be viewed at wileyonlinelibrary.com]

zoon at the end of 18 h for bacteria. Based on the results of the inhibition zone, data in Table 1 and Figure 2 displayed the inhibitory activities of the selected compounds. It has been observed that compounds **7**, **22**, **23**, and **24** exhibited a pronounced antimicrobial activity against all the tested microorganisms compared with the reference drugs, while compounds **3**, **4**, **6**, **14**, **18**, **20**, and **21** showed moderate activity. Compounds **13** and **19** showed low activity. The carboxylic acid moiety in thiazolidinone **7** increased both antibacterial and antifungal activities.

***In vitro* antiproliferative activity.** Anticancer activity screening of the synthesized compounds was measured *in vitro* using two different human cancer cell lines, namely, hepatocellular carcinoma (HepG-2) and mammary gland (MCF-7), using the standard MTT method [21]. Doxorubicin was selected as a standard reference anticancer agent. The chemosensitivity responses of cell lines to the new compounds are presented in Table 2. The results obtained indicated that the tested compounds exhibited good, moderate, or weak antiproliferative activities against the tested cell lines. In general, the compounds **7**, **23**, and **24** were found to be the most potent derivatives against the two cell lines. The other compounds were found to possess either moderate or weak antiproliferative activities against the two cell lines.

Table 2

Cytotoxic effect on human cell lines (HepG-2 and MCF-7).

	In vitro cytotoxicity IC_{50} (μg/mL)[a]	
Sample	HepG-2	MCF-7
Doxorubicin	5.42 ± 0.31	4.17 ± 0.47
3	78.03 ± 3.1	80.31 ± 2.2
4	70.55 ± 0.2	75.21 ± 1.8
6	81.15 ± 1.8	85.61 ± 1.2
7	4.21 ± 2.9	5.33 ± 0.3
8	64.23 ± 2.3	68.85 ± 4.1
14	50.12 ± 0.9	49.80 ± 0.9
15	67.62 ± 2.4	41.42 ± 0.3
18	67.75 ± 0.2	58.62 ± 0.2
20	45.84 ± 1.1	54.76 ± 0.6
23	5.94 ± 0.5	4.81 ± 0.7
24	5.61 ± 0.3	4.62 ± 0.2

[a]IC_{50} (lethal concentration of the sample that causes the death of 50% of cells in 48 h): 1–10 (*very strong*), 11–20 (*strong*), 21–50 (*moderate*), 51–100 (*weak*), above 100 (*noncytotoxic*).

Structure activity relationships.

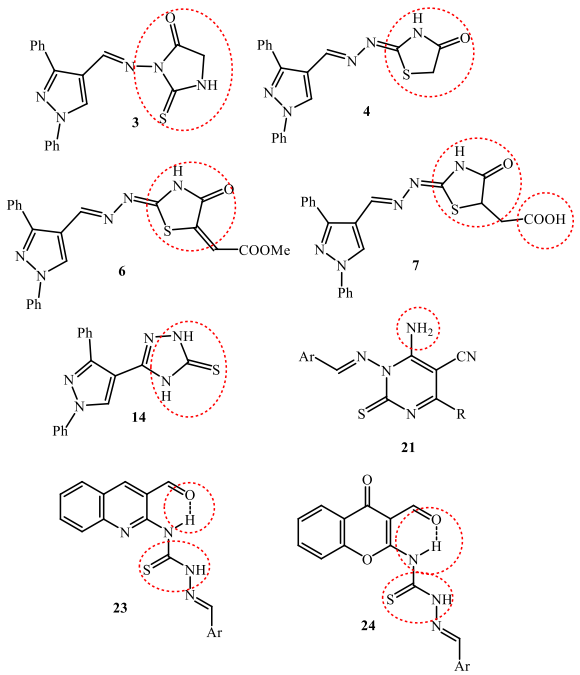


Figure 3. Structure activity relationships of the more potent compounds. [Color figure can be viewed at wileyonlinelibrary.com]

CONCLUSION

Transformation of 1,3-diphenylpyrazole-4-carboxaldehyde thiosemicarbazone into novel heterocycles, for example, thiazolidinone, imidazolidine, thiazole, and pyrimidine derivatives was carried out through a facile strategy and evaluated for antitumor as well as antimicrobial activities. The results showed that some of synthesized compounds have promising activities.

EXPERIMENTAL

Chemistry. All melting points were measured on a Gallenkamp electric melting point apparatus and are uncorrected. The Fourier transform infrared (FTIR) spectra were recorded using potassium bromide disks on Fourier Transform Infrared Thermo Electron Nicolet iS10 Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the Central Laboratory of Faculty of Science, Ain Shams University. The ^{1}H-NMR spectra were run at 400 *MHz* on a GEMINI 400 BB NMR Spectrometer (GEMINI, Manufacturing & Engineering Inc., Anaheim, CA, USA) using tetramethyl silane as internal standard in deuterated dimethylsulfoxide (DMSO-d_6) at the Main Defense Chemical Laboratory, Cairo. The mass spectra (MS) were recorded on a Shimadzu GC–MS-QP-1000EX mass spectrometer (Shimadzu Scientific Instruments, Inc., USA) operating at 70 eV at the Micro-analytical Center of Cairo University. The reactions were monitored by TLC using Merck Kiesel gel 60F_{254} analytical sheets obtained from Fluka. The biological activities were performed at Micro-analytical Center of Mansoura University, Egypt.

Formation of imidazolidine derivative 3. ***Method I: Reaction of thiosemicarbazone 2 with chloroacetic acid.*** A solution of pyrazolylthiosemicarbazone **2** (2 mmol) and chloroacetic acid (2 mmol) in pyridine (10 mL) was heated under reflux for 6 h and left overnight at room temperature. The precipitated solid was collected by filtration and recrystallized from ethanol/dioxane mixture (1:1) to afford imidazolidine derivative **3** as white crystals, mp 324–326°C, yield 74%.

Method II: Reaction of 2 with chloroacetyl chloride. To a stirred solution of **2** (2 mmol) in dry dioxane (10 mL) containing few drops of triethylamine, chloroacetyl chloride (2 mmol) was added dropwise at room temperature. Stirring was continued for 4 h. The reaction mixture was then poured onto ice/cooled water with stirring for 5 min. The precipitated solid was collected by filtration and recrystallized from ethanol/dioxane mixture (1:1) to give **3**, mp 324–326°C, yield 81%.

3-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)amino)-2-thioxoimidazolidin-4-one (3). IR (KBr) (ν, cm^{-1}): 3421 (*br*., OH, enol form, NH), 3052 (aryl-H), 2956 (alkyl-H), 1718 (C═O), 1640 (C═N), 1248 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 11.86 (s, 1H, NH, *D_2O-exchangeable*), 8.94 (s, 1H, CH═N), 8.43 (s, 1H, C—H pyrazolyl), 7.99–7.97 (d, 2H, Ph—C═N, J = 7.5 Hz), 7.87–7.85 (d, 2H, Ph—N, J = 8.1 Hz), 7.58–7.36 (m, 6H, Ar—H), 3.89 (s, 2H, CH_2). MS (*m/z*, %): 361 ($M^{+\cdot}$, 42). *Anal*. Calcd for $C_{19}H_{15}N_5OS$ (361.42): C, 63.14; H, 4.18; N, 19.38; S, 8.87. Found: C, 63.08; H, 4.09; N, 19.40; S, 8.81.

Formation of thiazolidinone derivative 4. ***Method I: Reaction of 2 with chloroacetic acid.*** A mixture of **2** (2 mmol), chloroacetic acid (2 mmol), and freshly prepared anhydrous sodium acetate (2 mmol) in an absolute ethanol (20 mL) was heated under reflux for 3 h. The separated solid while hot was collected by filtration and recrystallized from ethanol/dioxane (1:1) to afford thiazolidinone **4** as white crystals, mp 306–308°C, yield 79%.

Method II: Reaction of 2 with ethyl chloroacetate. To a solution of **2** (2 mmol) and anhydrous sodium acetate (2 mmol) in an absolute ethanol (20 mL), ethyl chloroacetate (2 mmol) was added. The reaction mixture was heated under reflux for 2 h. The precipitated solid while hot was collected by filtration and recrystallized from ethanol/dioxane (1:1) to afford **4**, mp 306–308°C, yield 83%.

2-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)hydrazono) thiazolidin-4-one (4). IR (KBr) (ν, cm^{-1}): 3435 (*br*., OH, lactim form), 3052 (aryl-H), 2961 (alkyl-H), 1717 (C═O), 1640 (C═N). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 12.05 (s, 1H, NH, *D_2O-exchangeable*), 8.96 (s, 1H, CH═N), 8.50 (s, 1H, C—H pyrazolyl), 7.98–7.96 (d, 2H, Ph—C═N, J = 7.5 Hz), 7.86–7.84 (d, 2H, Ph—N, J = 8.1 Hz), 7.57–7.36 (m, 6H, Ar—H), 3.95 (s, 2H, CH_2). MS (*m/z*, %): 361 ($M^{+\cdot}$, 33). *Anal*. Calcd for $C_{19}H_{15}N_5OS$ (361.42): C, 63.14; H, 4.18; N, 19.38; S, 8.87. Found: C, 63.03; H, 4.13; N, 19.35; S, 8.84.

Aldol condensation of thiazolidinone 4 with *p*-nitrobenzaldehyde. Equimolar mixture of thiazolidinone derivative **4**, *p*-nitrobenzaldehyde, and anhydrous sodium acetate (2 mmol) in glacial acetic acid (10 mL) was heated under reflux for 5 h. The separated solid while hot was collected by filtration and recrystallized from dioxane to give **5b**, yield 51%. The residual mother liquor, after the separation of **5b**, was concentrated and then left to cool at room temperature. The obtained precipitate was filtered off and recrystallized from ethanol/dioxane mixture (1:1) to afford **5a**, yield 43%.

2-((-(1,3-Diphenyl-1H-pyrazol-4-yl)methylene)hydrazono)-5-(-4-nitrobenzylidene)-thiazolidin-4-one (5a). Yellow crystals, mp 258–260°C. IR (KBr) (ν, cm^{-1}): 3425 (*br*., OH, lactim form), 3051 (aryl-H), 1717 (C═O), 1640 (C═N). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 11.89 (s, 1H, NH,

D_2O-exchangeable), 8.94 (s, 1H, CH═N), 8.42 (s, 1H, C─H pyrazolyl), 8.56–8.54 (d, 2H, *p*-nitrophenyl, *J* = 8.1 Hz), 8.33–8.31 (d, 2H, *p*-nitrophenyl, *J* = 8.0 Hz), 7.99 (s, 1H, CH═), 7.96–7.36 (m, 10H, Ar─H). MS (*m/z*, %): 494 ($M^{+\cdot}$, 24). *Anal*. Calcd for $C_{26}H_{18}N_6O_3S$ (494.52): C, 63.15; H, 3.67; N, 16.99; S, 6.48. Found: C, 63.04; H, 3.61; N, 17.01; S, 6.41.

3-Acetyl-2-(((1,3-diphenyl-1H-pyrazol-4-yl)methylene) hydrazono)-5-(-4-nitrobenzylidene)-thiazolidin-4-one (5b). Yellow crystals, mp 310–312°C. IR (KBr) (*v*, cm^{-1}): 3052 (aryl-H), 2938 (alkyl-H), 1716, 1673 (C═O), 1639 (C═N). ^{1}H-NMR (DMSO-d_6): (*E*-isomer and *Z*-isomer, 80:20%) δ_H (ppm) 10.18 and 9.99 (two singlets, 1H, CH═N), 9.38 and 9.31 (two singlets, 1H, C─H pyrazolyl), 8.93 and 8.47 (two singlets, 1H, CH═), 8.45–8.42 (d, 2H, *p*-nitrophenyl, *J* = 8.7 Hz), 8.29–8.27 (d, 2H, *p*-nitrophenyl, *J* = 8.6 Hz), 8.01–7.38 (m, 10H, Ar─H), 1.91 (s, 3H, CH_3). MS (*m/z*, %): 536 ($M^{+\cdot}$, 27). *Anal*. Calcd for $C_{28}H_{20}N_6O_4S$ (536.56): C, 62.68; H, 3.76; N, 15.66; S, 5.98. Found: C, 62.62; H, 3.73; N, 15.69; S, 5.91.

Reaction of thiosemicarbazone 2 with dimethyl acetylenedicarboxylate. A solution of **2** (2 mmol) and dimethyl acetylenedicarboxylate (2 mmol) in methanol (20 mL) was heated under reflux for 1 h. The precipitated solid while hot was collected by filtration and recrystallized from methanol/dioxane mixture (2:1) to afford thiazolidinone derivative **6**.

Methyl 2-(-2-((-(1,3-diphenyl-1H-pyrazol-4-yl)methylene) hydrazono)-4-oxothiazolidin-5-ylidene) acetate (6). Yellow crystals, mp 300–302°C, yield 82%. IR (KBr) (*v*, cm^{-1}): 3446 (*br*., OH, lactim form), 3056 (aryl-H), 2986, 2954 (alkyl-H), 1713 (C═O ester), 1700 (C═O thiazolidinone), 1636 (C═N). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 12.78 (s, 1H, NH, *D_2O-exchangeable*), 9.03 (s, 1H, CH═N), 8.52 (s, 1H, C─H pyrazolyl), 8.01–7.98 (d, 2H, Ph─C═N, *J* = 8.4 Hz), 7.89–7.86 (d, 2H, Ph─N, *J* = 7.5 Hz), 7.59–7.39 (m, 6H, Ar─H), 6.66 (s, 1H, CH═), 3.81 (s, 3H, CH_3). MS (*m/z*, %): 431 ($M^{+\cdot}$, 54). *Anal*. Calcd for $C_{22}H_{17}N_5O_3S$ (431.47): C, 61.24; H, 3.97; N, 16.23; S, 7.43. Found: C, 61.21; H, 3.92; N, 16.29; S, 7.38.

Reaction of thiosemicarbazone 2 with maleic anhydride. A suspension of **2** (2 mmol) and maleic anhydride (2 mmol) in dry toluene (15 mL) was heated under reflux for 4 h. The reaction mixture was concentrated and cooled to room temperature. The obtained solid was collected by filtration and recrystallized from ethanol/dioxane mixture (1:1) to afford thiazolidinone derivative **7**.

2-(2-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene) hydrazono)-4-oxothiazolidin-5-yl) acetic acid (7). White crystals, mp 260–262°C, [Lit. [18] mp 236–238°C].

Reaction of thiosemicarbazone 2 with 3′-nitro-ω-bromoacetophenone. A solution of **2** (2 mmol) and 3′-nitro-ω-bromoacetophenone (2 mmol) in an absolute ethanol (15 mL) containing anhydrous sodium acetate (2 mmol) was heated under reflux for 1 h. The precipitated solid while hot was filtered off and recrystallized from ethanol to afford thiazole derivative **8**.

2-(2-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)hydrazinyl)-4-(3-nitrophenyl) thiazole (8). Yellow crystals, mp 198–200°C, yield 81%. IR (KBr) (*v*, cm^{-1}): 3396 (NH), 3058 (aryl-H), 1628 (C═N). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 12.06 (s, 1H, NH, *D_2O-exchangeable*), 8.89 (s, 1H, CH═N), 8.66 (s, 1H, *m*-nitrophenyl), 8.18 (s, 1H, C─H pyrazolyl), 8.29–8.27 (d, 1H, *m*-nitrophenyl, *J* = 7.8 Hz), 8.15–8.13 (d, 1H, *m*-nitrophenyl, *J* = 8.4 Hz), 8.00–7.97 (d, 2H, Ph─C═N, *J* = 8.1 Hz), 7.81–7.79 (d, 2H, Ph─N, *J* = 8.1 Hz), 7.72–7.67 (dd, 1H, *m*-nitrophenyl, *J* = 8.1 & 7.8 Hz), 7.61 (s, 1H, C─H thiazolyl), 7.57–7.35 (m, 6H, Ar─H). MS (*m/z*, %): 466 ($M^{+\cdot}$, 71). *Anal*. Calcd for $C_{25}H_{18}N_6O_2S$ (466.52): C, 64.37; H, 3.89; N, 18.01; S, 6.87. Found: C, 64.31; H, 3.83; N, 18.10; S, 6.82.

Reaction of thiazolidinone 7 with *o*-phenylenediamine and/or *o*-aminothiophenol. A solution of thiazolidinone-5-acetic acid **7** (2 mmol) and *o*-phenylenediamine or *o*-aminothiophenol (2 mmol) in methanol (15 ml) containing few drops of conc. HCl was heated under reflux for 1 h. The separated solid while hot was filtered off and recrystallized from dioxane to afford benzimidazole **10a** and benzothiazole **10b** derivatives, respectively. These compounds were identical in all respects (IR, ^{1}HNMR, mp, mixed mp and TLC) with authentic samples prepared by reacting of equimolar mixture of 2-(2,4-dioxothiazolidin-5-yl) acetic acid **11** with *o*-phenylenediamine or *o*-aminothiophenol in methanol/HCl.

5-((1H-benzo[d]imidazol-2-yl)methyl)thiazolidine-2,4-dione (10a). White crystals, mp 244–246°C, yield 71%. [Lit. [19] mp 244–246°C].

5-(Benzo[d]thiazol-2-ylmethyl)thiazolidin-2,4-dione (10b). Yellow crystals, mp 172–174°C, yield 79%. [Lit. [19] mp 172–174°C].

Reaction of thiazolidinone (7) with thionyl chloride. A solution of thiazolidinone-5-acetic acid **7** (2 mmol) in thionyl chloride (5 mL) was heated at 60°C for 5 h. The reaction mixture was evaporated under vacuum. The remained residue was recrystallized from dioxane to produce furothiazole derivative **13**.

2-((-(1,3-Diphenyl-1H-pyrazol-4-yl)methylene)hydrazono) furo[2,3-d]thiazol-5(2H)-one (13). Yellow crystals, mp >360°C, yield 43%. IR (KBr) (*v*, cm^{-1}): 3058 (aryl-H), 1734 (C═O *α,β*-unsaturated furanone), 1621 (C═N). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 8.90 (s, 1H, CH═N), 8.41 (s, 1H, C─H pyrazolyl), 7.92–7.38 (m, 10H, Ar─H), 7.34 (s, 1H, CH═). MS (*m/z*, %): 399 ($M^{+\cdot}$, 18).

Anal. Calcd for $C_{21}H_{13}N_5O_2S$ (399.43): C, 63.15; H, 3.28; N, 17.53; S, 8.03. Found: C, 63.11; H, 3.22; N, 17.61; S, 7.98.

Ring closure of thiosemicarbazone 2 using pyridine. A solution of **2** (2 mmol) in pyridine (10 mL) was heated under reflux for 6 h and then left to cool at room temperature. The obtained precipitate was collected by filtration and recrystallized from dioxane to afford mercaptotriazole derivative **14**.

5-(1,3-Diphenyl-1H-pyrazol-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (14). White crystals, mp 246–248°C, yield 59%. IR (KBr) (ν, cm^{-1}): 3390 (NH), 3052 (aryl-H), 1617 (C═N), 1213 (C═S). ^{1}H-NMR (DMSO-d_6): (thiolactam and thiolactim isomers, 1:1) δ_H (ppm) 9.30 (s, 1H, NH, *D_2O-exchangeable*), 9.12 and 8.67 (two singlets, 1H, C—H pyrazolyl), 8.85 (1H, SH, *D_2O-exchangeable*), 8.55 (s, 1H, NH, *D_2O-exchangeable*), 8.02–7.99 (d, 2H, Ph—C═N, J = 8.1 Hz), 7.87–7.84 (d, 2H, Ph—N, J = 8.4 Hz) 7.82–7.36 (m, 6H, Ar—H). MS (*m/z*, %): 319 ($M^{+\cdot}$, 42). *Anal*. Calcd for $C_{17}H_{13}N_5S$ (319.39): C, 63.93; H, 4.10; N, 21.93; S, 10.04. Found: C, 63.81; H, 3.98; N, 21.85; S, 9.98.

Ring closure of thiosemicarbazone 2 using HCl/AcOH. Thiosemicarbazone **2** (2 mmol) was added to equimolar mixture of HCl/CH_3COOH (10 mL), and then the reaction mixture was heated under reflux 4 h. The reaction mixture was cooled and poured onto ice-cooled water. The precipitated solid was collected by filtration and recrystallized from ethanol to afford the fused heterocycle derivative **15**.

1,3-Diphenyl-1,6-dihydropyrazolo[3,4-*c*]pyrazole (15). Greenish yellow crystals, mp 238–240°C, yield 62%. IR (KBr) (ν, cm^{-1}): 3343 (*br*., NH), 3064 (aryl-H), 1618 (C═N). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 13.20 (s, 1H, NH, exchangeable with D_2O), 8.40 (CH═N), 7.90–7.42 (m, 10H, two phenyl). MS (*m/z*, %): 260 ($M^{+\cdot}$, 13). *Anal*. Calcd for $C_{16}H_{12}N_4$ (260.30): C, 73.83; H, 4.65; N, 21.52. Found: C, 73.71; H, 4.52; N, 21.61.

Hydrazinolysis of thiosemicarbazone 2. Hydrazine hydrate (2.2 mmol, 80%) was added to solution of thiosemicarbazone **2** (2 mmol) in absolute ethanol (20 mL) and heated under reflux for 6 h. The reaction mixture was concentrated and then left to cool at room temperature. The obtained colorless crystals were collected by filtration and found to be thiosemicarbazide [mp, mixed mp, TLC, IR]. The residual part was recrystallized from benzene/ethanol mixture (1:1) and found to be diheterylhydrazine **17**. The latter compound was identical in all respects (IR, mp, mixed mp, and TLC) with an authentic sample prepared by the condensation of 1,3-diphenylpyrazole-4-carboxaldehyde **1** with hydrazine hydrate in ethanolic solution [8].

1,2-Bis((1,3-diphenyl-1H-pyrazol-4-yl)methylene) hydrazine (17). White crystals, mp 222–224°C, yield 71%. [Lit. [8] mp 220–222°C].

Reaction of 2 with malonic acid. A mixture of **2** (2 mmol) and malonic acid (2 mmol) in acetylchloride (5 mL) was heated on water bath at 55°C for 4 h. The reaction mixture was then cooled and poured onto ice-cold water with stirring. The yellow precipitated solid was collected by filtration, dried, and then recrystallized from ethanol/dioxane mixture (2:1) to afford pyrimidinone **18**.

3-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)amino)-6-oxo-2-thioxo-1,2,3,6-tetrahydro-pyrimidin-4-yl acetate (18). Yellow crystals, mp 338–340°C, yield 68%. IR (KBr) (ν, cm^{-1}): 3421 (OH, lactim form), 3257 (NH, lactam form), 3061 (aryl-H), 2978 (alkyl-H), 1721 (C═O ester), 1672 (C═O pyrimdinone), 1600 (C═N), 1213 (C═S). ^{1}H-NMR (DMSO-d_6): (*E*-isomer and *Z*-isomer, 1:1) δ_H (ppm) 12.86 (*br*. s, 1H, OH, lactim form, *D_2O-exchangeable*), 10.84 and 10.79 (two singlets, 1H, NH, *D_2O-exchangeable*), 9.88 and 9.77 (two singlets, 1H, CH═N), 8.31 and 8.26 (two singlets, 1H, C—H pyrazolyl), 7.97–7.49 (m, 10H, Ar—H), 7.36 (s, 1H, C—H pyrimidine), 2.03 and 1.97 (two singlets, 3H, CH_3). MS (*m/z*, %): 431 ($M^{+\cdot}$, 25). *Anal*. Calcd for $C_{22}H_{17}N_5O_3S$ (431.47): C, 61.24; H, 3.97; N, 16.23; S, 7.43. Found: C, 61.15; H, 3.81; N, 16.18; S, 7.34.

General procedure for the reaction of 2 with acetylacetone, ethyl benzoylacetate, and/or arylidene malononitrile. A solution of thiosemicarbazone **2** (2 mmol) and acetylacetone, ethyl benzoylacetate, or 2-(4-methoxybenzylidene) malononitrile (2 mmol) in absolute ethanol (20 mL) was refluxed in the presence of sodium ethoxide (0.05 g Na in 20-mL ethanol) for 8–10 h. The whole mixture was then cooled and poured onto ice/HCl. The precipitated solid was filtered off, dried, and then recrystallized from ethanol to furnish pyrimidine derivatives **19**–**21**, respectively.

1-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)amino)-4,6-dimethylpyrimidine-2(1H)-thione (19). Faint brown crystals, mp 241–243°C, yield 52%. IR (KBr) (ν, cm^{-1}): 3056 (aryl-H), 2972 (alkyl-H), 1619 (C═N), 1220 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 8.90 (s, 1H, CH═N), 8.40 (s, 1H, C—H pyrazolyl), 7.81–7.45 (m, 10H, Ar—H), 7.02 (s, 1H, C—H pyrimidine), 2.25 and 2.22 (two singlets, 6H, two CH_3). MS (*m/z*, %): 385 ($M^{+\cdot}$, 33). *Anal*. Calcd for $C_{22}H_{19}N_5S$ (385.49): C, 68.55; H, 4.97; N, 18.17; S, 8.32. Found: C, 68.50; H, 4.94; N, 18.20; S, 8.27.

1-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)amino)-6-hydroxy-4-phenylpyrimidine-2(1H)-thione (20). Beige crystals, mp 236–238°C, yield 59%. IR (KBr) (ν, cm^{-1}): 3444 (*br*. OH), 3062 (aryl-H), 1618 (C═N), 1261 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 11.20 (*br*. s, 1H, OH, *D_2O-exchangeable*), 9.18 (s, 1H, CH═N), 8.20 (s, 1H, C—H pyrazolyl), 7.85–7.37 (m, 15H, Ar—H), 7.01 (s,

1H, C—H pyrimidine). MS (*m/z*, %): 449 ($M^{+\cdot}$, 52). *Anal*. Calcd for $C_{26}H_{19}N_5OS$ (449.53): C, 69.47; H, 4.26; N, 15.58; S, 7.13. Found: C, 69.42; H, 4.18; N, 15.61; S, 7.08.

6-Amino-1-(((1,3-diphenyl-1H-pyrazol-4-yl)methylene) amino)-4-(4-methoxyphenyl)-2-thioxo-1,2-dihydropyrimidine-5-carbonitrile (21). Faint brown crystals, mp 270–272°C, yield 44%. IR (KBr) (*v*, cm^{-1}): 3326, 3256 (NH_2), 3062 (aryl-H), 2971 (alkyl-H), 2216 (C≡N), 1618 (C═N), 1220 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 6.50 (s, 2H, NH_2, *D_2O-exchangeable*), 9.20 (s, 1H, CH═N), 8.31 (s, 1H, C—H pyrazolyl), 7.91–7.88 (d, 2H, *p*-anisyl, *J* = 8.8 Hz), 7.83–7.81 (d, 2H, Ph—C═N, *J* = 8.6 Hz), 7.68–7.66 (d, 2H, Ph—N, *J* = 8.5 Hz), 7.03–7.01 (d, 2H, *p*-anisyl, J = 8.8 Hz), 7.55–7.36 (m, 6H, Ar—H), 3.75 (s, 3H, CH_3). MS (*m/z*, %): 503 ($M^{+\cdot}$, 23). *Anal*. Calcd for $C_{28}H_{21}N_7OS$ (503.58): C, 66.78; H, 4.20; N, 19.47; S, 6.37. Found: C, 66.72; H, 4.15; N, 19.51; S, 6.32.

Condensation of 2 with ethyl cyanoacetate and *p*-anisaldehyde. Equimolar mixture of **2**, ethyl cyanoacetate, and *p*-anisaldehyde (2 mmol) in absolute ethanol (20 mL) was treated with sodium ethoxide (0.05 g Na in 20-mL ethanol). The whole mixture was heated under reflux for 6 h. The obtained solid during heating was filtered off and recrystallized from ethanol to afford hydroxypyrimidine derivative **22**.

1-(((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)amino)-6-hydroxy-4-(4-methoxyphenyl)-2-thioxo-1,2-dihydropyrimidine-5-carbonitrile (22). Beige crystals, mp 254–256°C, yield 67%. IR (KBr) (*v*, cm^{-1}): 3445 (*br*., OH), 3063 (aryl-H), 2972 (alkyl-H), 2215 (C≡N), 1627 (C═N), 1262 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 11.31 (*br*. s, 1H, OH, *D_2O-exchangeable*), 9.17 (s, 1H, CH═N), 8.21 (s, 1H, C—H pyrazolyl), 7.90–7.87 (d, 2H, *p*-anisyl, *J* = 8.8 Hz), 7.84–7.82 (d, 2H, Ph—C═N, *J* = 8.7 Hz), 7.67–7.65 (d, 2H, Ph—N, *J* = 8.5 Hz), 7.03–7.01 (d, 2H, *p*-anisyl, *J* = 8.8 Hz), 7.56–7.37 (m, 6H, Ar—H), 3.79 (s, 3H, CH_3). MS (*m/z*, %): 504 ($M^{+\cdot}$, 41). *Anal*. Calcd for $C_{28}H_{20}N_6O_2S$ (504.57): C, 66.65; H, 4.00; N, 16.66; S, 6.35. Found: C, 66.61; H, 3.95; N, 16.69; S, 6.30.

Reaction of 2 with 2-chloroquinoline-3-carboxaldehyde. 2-Chloroquinoline-3-carboxaldehyde (2 mmol) was treated with a solution of thiosemicarbazone **2** (2 mmol) in glacial acetic acid (10 mL). The reaction mixture was heated under reflux for 8 h. The yellow precipitated solid after cooling was filtered off and recrystallized from dioxane to give the quinoline derivative **23**.

2-((1,3-Diphenyl-1*H*-pyrazol-4-yl)methylene)-*N*-(3-formylquinolin-2-yl)hydrazine-1-carbothioamide (23). Yellow crystals, mp 263–265°C, yield 52%. IR (KBr) (*v*, cm^{-1}): 3415 (*br*., NH, H-bonding), 3057 (aryl-H), 1662 (C═O), 1622 (C═N), 1216 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 11.30 (s, 1H, NH, *D_2O-exchangeable*), 10.50 (s, 1H, NH, *D_2O-exchangeable*), 9.75 (s, 1H, CHO), 8.55 (s, 1H, CH═N), 8.34 (s, 1H, C—H pyrazolyl), 8.20 (s, 1H, C—H quinoline), 7.88–7.71 (m, 4H, quinoline), 7.68–7.38 (m, 10H, two phenyl). MS (*m/z*, %): 476 ($M^{+\cdot}$, 15). *Anal*. Calcd for $C_{27}H_{20}N_6OS$ (476.56): C, 68.05; H, 4.23; N, 17.64; S, 6.73. Found: C, 68.01; H, 4.17; N, 17.70; S, 6.68.

Reaction of 2 with chromone-3-carboxaldehyde. A solution of thiosemicarbazone **2** (2 mmol), chromone-3-carboxaldehyde (2 mmol) in absolute ethanol (20 mL) was heated under reflux for 5 h. The precipitated solid while heating was collected by filtration and recrystallized from dioxane to give the chromone derivative **24**.

2-((1,3-Diphenyl-1H-pyrazol-4-yl)methylene)-N-(3-formyl-4-oxo-4H-chromen-2-yl)-hydrazine-1-carbothioamide (24). Brown crystals, mp 234–236°C, yield 69%. IR (KBr) (*v*, cm^{-1}): 3427, 3331, 3258 (2 NH, H-bonding), 3059 (aryl-H), 1680 (C═O aldehyde), 1648 (C═O chromone), 1619 (C═N), 1219 (C═S). ^{1}H-NMR (DMSO-d_6): δ_H (ppm) 11.35 (s, 1H, NH, *D_2O-exchangeable*), 10.81 (s, 1H, NH, *D_2O-exchangeable*), 9.95 (s, 1H, CHO), 8.62 (s, 1H, CH═N), 8.41 (s, 1H, C—H pyrazolyl), 7.95–7.67 (m, 4H, chromone), 7.63–7.38 (m, 10H, two phenyl). MS (*m/z*, %): 493 ($M^{+\cdot}$, 33). *Anal*. Calcd for $C_{27}H_{19}N_5O_3S$ (493.54): C, 65.71; H, 3.88; N, 14.19; S, 6.50. Found: C, 65.59; H, 3.73; N, 14.27; S, 6.39.

Biological assay. ***Antimicrobial activity. Materials and methods.*** Antimicrobial activity of the tested samples was determined using a modified Kirby–Bauer disc diffusion method [20]. Briefly, 100 µL of the test bacteria/fungi were grown in 10 mL of fresh media until they reached a count of 108 cells/mL for bacteria or 105 cells/mL for fungi approximately [22]. A 100 µL of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

Isolated colonies of each organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility by disc diffusion method [23]. Of the many media available, National Committee for Clinical Laboratory Standards recommends Mueller–Hinton agar due to its results in good batch-to-batch reproducibility. Disc diffusion method for filamentous fungi tested by using approved standard method (M38-A) developed [24] for evaluating the susceptibilities of filamentous fungi to antifungal agents. Disc diffusion method for yeasts developed by using approved standard method (M44-P) [25]. Plates inoculated with filamentous fungi as *Aspergillus flavus* at 25°C for 48 h; Gram (+) bacteria as *Staphylococcus aureus, Bacillus subtilis*; Gram (−) bacteria as *Escherichia coli, Pseudomonas aeuroginosa* they were incubated at 35–37°C for 24–48 h and yeast as *Candida albicans* incubated at 30°C for 24–48 h and then the diameters of the inhibition zones were measured in millimeters [21].

Standard discs of Ampicillin (antibacterial agent), Amphotericin B (antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 μL of solvent (distilled water, chloroform, DMSO) were used as a negative control. The agar used is Meuller–Hinton agar that is rigorously tested for composition and pH. Further, the depth of the agar in the plate is a factor to be considered in the disc diffusion method. This method is well documented, and standard zones of inhibition have been determined for susceptible and resistant values.

Blank paper disks (Schleicher and Schuell, Spain) with a diameter of 8.0 mm were impregnated 10 μL of tested concentration of the stock solutions. When a filter paper disc impregnated with a tested chemical is placed on agar, the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar, it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as "Zone of inhibition" or "Clear zone."

For the disc diffusion, the zone diameters were measured with slipping calipers of the National Committee for Clinical Laboratory Standards [26]. Agar-based methods such as Etest ad disk diffusion can be good alternatives because they are simpler and faster than broth-based methods [27,28].

Cytotoxicity assay. ***Cell line.*** The cytotoxic activity of the synthesized compounds was tested against two human tumor cell lines, namely, hepatocellular carcinoma HepG-2 and mammary gland breast cancer MCF-7. The cell lines were obtained from ATCC through Holding company for biological products and vaccines, Cairo, Egypt. Doxorubicin was used as a standard anticancer drug for comparison.

Chemical reagents. The reagents used were RPMI-1640 medium, MTT, DMSO, Doxorubicin (Sigma Co., St. Louis, USA), and Fetal Bovine serum (GIBCO, Paisley, UK).

MTT assay. The different cell lines mentioned earlier were utilized to measure the inhibitory effects of compounds on cell growth using the MTT assay [29–31]. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. The cell lines were seeded in a 96-well plate at a density of 1.0 × 104 cells/well [32] at 37°C for 48 h under 5% CO_2. After incubation, the cells treated with different concentrations of compounds and incubated for 24 h. After 24 h of drug treatment, 20 μl of MTT solution at 5 mg/ml was added and then incubated for 4 h. DMSO in volume of 100 μL is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570 nm using a plate reader (EXL 800, Bio-Tech, Winoosky, VT, USA). The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated sample) × 100.

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