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Original article

Synthesis, antimicrobial, antioxidant, anti-hemolytic and cytotoxic evaluation of new imidazole-based heterocycles

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ABSTRACT

In the present work, 1-(5-methyl-2-phenyl-1*H*-imidazol-4-yl)ethanone **1** was prepared and used as a precursor for the synthesis of new thiazole, arylidiene and coumarin derivatives. The antimicrobial, antioxidant, anti-hemolytic, and cytotoxic activities of new compounds have been screened. Compound **12** showed an excellent antibacterial activity for all the tested bacteria with minimal inhibitory concentration (MIC) ranged between 21.9 and 43.8 μg/mL. While, compounds **2**, **8** and **10a** were the best antioxidant reagents using the DPPH method. Compounds **6a** and **10b** proved to exhibit potent anti-oxidative activity as reflected in the ability to inhibit lipid per-oxidation in rat brain and kidney homogenates and rate erythrocyte hemolysis. Compounds **6a** proved to have the highest cytotoxic activity (81.9%) followed by **2**, **6c**, **7b** and **12** using in vitro Ehrlich ascites assay. The details synthetic methods, spectroscopic data and biological results are recorded.

1. Introduction

In recent years, the high therapeutic properties of the imidazole related drugs have been attracting the attention the medicinal chemists to synthesize a large number of novel chemotherapeutic agents. Imidazole drugs have broadened scope in remedying various dispositions in clinical medicines. Medicinal properties of imidazole containing compounds include anticancer [1], antimicrobial [2,3], antibacterial [4], antifungal [5], and antioxidant [6]. Encouraged by these observations and in continuation of our previous work to discover new biologically active heterocyclic compounds [7–12], we synthesized newer heterocyclic imidazole derivatives, by facile and routine methods, with the hope to get better antimicrobial, antioxidant, and anti-hemolytic agents.

2. Results and discussion

2.1. Chemistry

For the synthesis of the title compounds, 2-(1-(5-methyl-2-phenyl-1*H*-imidazol-4-yl)ethylidene)hydrazinecarbothioamide **2** required as starting material and was prepared by the reaction of 4-acetyl-5-methyl-2-phenylimdazole **1** [13] with thiosemicarbazide. The target compounds **6**–**8** were obtained by reaction of equimolar quantities of thiosemicarbazide **2** with phenacyl bromides **3a**–**c**, hydrazonoyl chlorides **4a**, **b** or 2,3-dichloroquinoxaline **5** (Scheme 1).

The ^{1}H NMR spectra data were consistent with the assigned structures; thiazolidine CH_2 protons of **6** was observed as a broad singlet around δ 4.01 ppm, Also compound **7b** has three methyl groups appeared at 2.24, 2.88 and 3.24 ppm respectively, all the other aromatic and aliphatic protons were observed at the expected regions. The ^{13}C NMR of compound **6b** showed five carbon atoms bonded with nitrogen atom with double bond appeared at δ 142.45, 146.94, 152.27, 154.59, 170.35 ppm. The mass spectra of the new compounds are in agreement with their molecular formulas. All new compounds gave satisfactory elemental analysis.

4-Thiazolidinone compound **9** was obtained by reaction of thiosemicarbazide **2** with chloroacetic acid in glacial acetic acid and in the presence of anhydrous sodium acetate. Reaction of the latter product **9** with 4-flurobenzaldehyde and substituted pyrazole aldehydes afforded the corresponding arylidines **10**. The one pot synthesis of products **10** has been preceded via reaction of thiazolidin-4-one **9** with aldehydes in glacial acetic acid and presence of excess anhydrous sodium acetate (Scheme 2).

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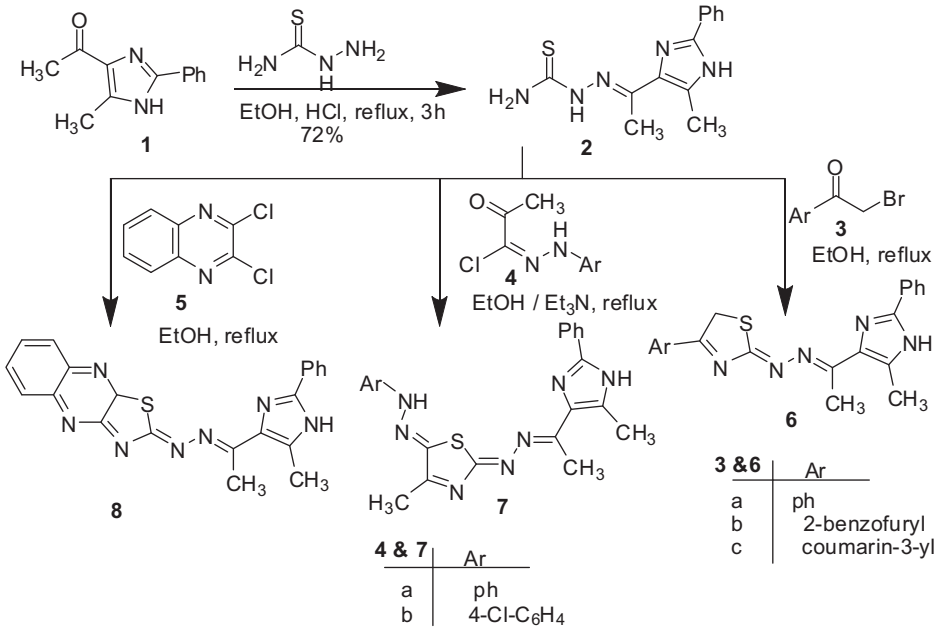


Scheme 1.

The ^{1}H NMR spectra data were also consistent with the assigned structures; thiazolidinone CH_2 protons of **9** appeared at δ 3.94 pm, arylidiene CH proton of **10c** was observed at 8.81 ppm. The carbonyl group of thiazolidinone derivative **10b** appeared at δ 146.09 ppm in its ^{13}C NMR.

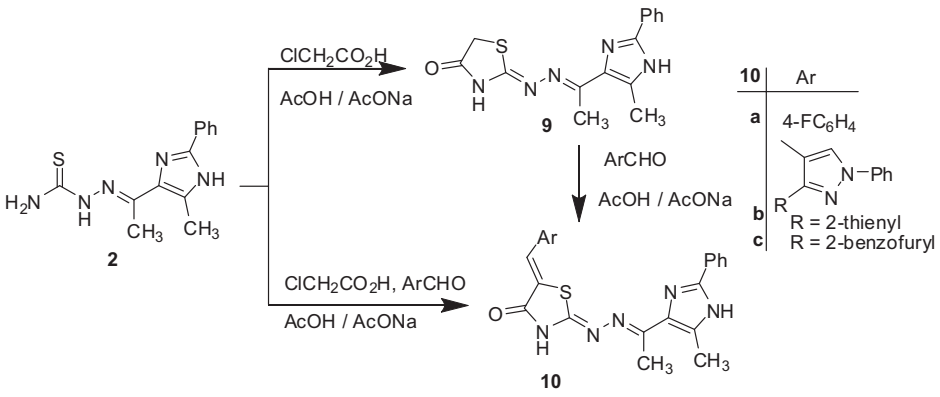
Cyanoacetylhydrazine reacts with 4-acetyl-5-methyl-2-phenyl-imdazole **1** to give the hydrazide–hydrazone derivative **11** (Scheme 3). The structure of compound **11** was established on the basis of analytical and spectral data. Thus ^{1}H NMR spectrum showed the presence of a singlet at δ 4.08 ppm for CH_2 group, and a singlet at δ 12.99 ppm for an NH group. The reaction of **11** with aromatic aldehydes either 4-flurobenzaldehyde, or pyrazole–aldehydes **13a**, **b** gave the benzalidene derivatives **12** and **14** (Scheme 3). The reaction of **11** with 4-hydroxysalicylaldehyde gave the coumarin derivative **15**, the reaction goes in analogy with the reported literature [14,15]. The ^{13}C NMR of compound **15** showed one carbonyl group at δ 163.10 ppm. Elemental analysis data revealed that variation in experimental values compared with calculated values is within ±0.4% (Table 1).

2.2. Biological activities

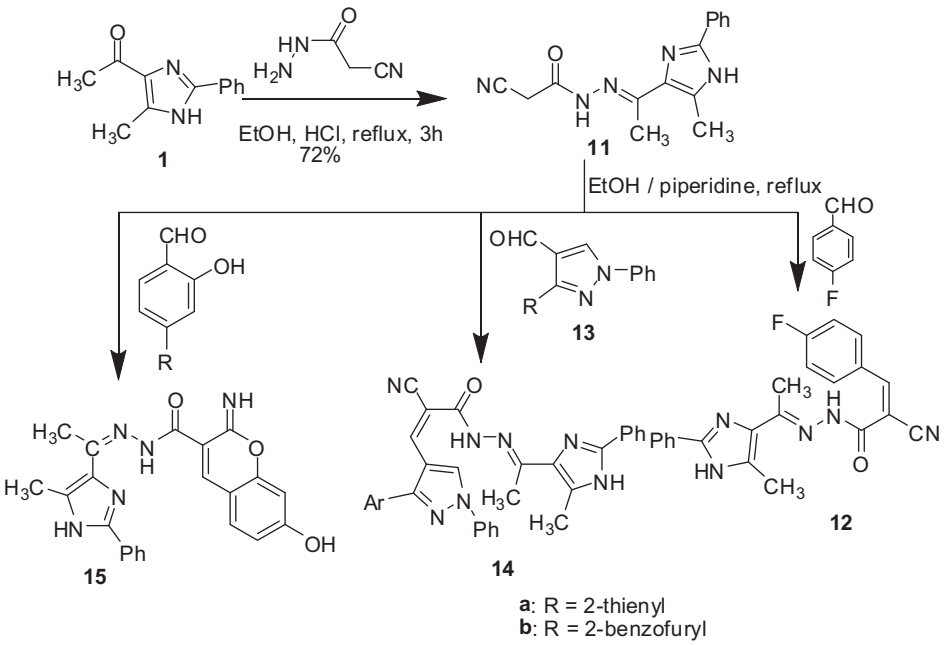
2.2.1. Antimicrobial activity

The antimicrobial activities of the new synthesized compounds against target pathogens were examined qualitatively and quantitatively by the presence or absence of inhibition zones and zone diameter. Results given in Table 2 showed that compound **12** showed an excellent antibacterial activity for all the tested bacteria with minimal inhibitory concentration (MIC) ranged between 21.9 and 43.8 μg/mL (Table 3). Moreover compounds **2**, **6a** and **9** showed moderate antibacterial activities against some Gram+ve and Gram−ve bacteria with minimal inhibitory (MIC) concentration of these compounds ranged from 21.9 to 87.5 μg/mL (Table 2). None of the tested compound showed antifungal activities against the tested fungus stains and yeast.

2.2.1.1. Structure–activity relationship (SAR) studies. The antimicrobial activity of the synthesized substituted imidazole derivatives is due to the presence of:



Scheme 2.



Scheme 3.

- Thiosemicarbazide fragment (compound **2**) and fluorinated arylidiene (compounds **12**)
- Thiazole ring (compound **6a** and **9**).

2.2.2. Antioxidant activity

2.2.2.1. DPPH free radical scavenging activity. Since the main mechanism of antioxidant action in foods is radical-scavenging, many methods have been developed in which the antioxidant activity is evaluated by the scavenging of synthetic radicals in polar organic solvents such as methanol at room temperature. In this study, the DPPH method was selected to evaluate the antioxidant activity of new compounds because it is one of the most effective methods for evaluating the concentration of radical-scavenging materials active by a chain-breaking mechanism [16]. The DPPH radical is a stable free radical and the DPPH radical-scavenging activity was determined by the decrease in absorbance at 517 nm, due to reduction by the antioxidant (AH) or reaction with a radical species, as shown in the Eq. (1) [17].

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH - R \quad (1)$$

We found that most of compounds showed considerable free radical-scavenging activities (Table 4). Compounds **2**, **8** and **10a** was the strongest radical scavenger among fungal isolates with CI_{50} 6.25 mg/mL, followed by compound **9** with CI_{50} 12.5 mg/mL while **6a**, **6b**, **7a** and **7b** with CI_{50} 25 mg/mL. However the other compounds were moderate radical scavengers with CI_{50} ranged between 100 and 200 mg/mL.

2.2.2.2. Antioxidant activity using ABTS inhibition and erythrocyte hemolysis. All compounds were tested for antioxidant activity as reflected in the ability to inhibit lipid per-oxidation in rat brain and kidney homogenates and rate erythrocyte hemolysis. The pro-oxidant activities of the compounds were assayed for their anti-oxidant effects using ABTS assay. Compounds **6a** and **10b** proved to exhibit potent antioxidative activity. On the other hand, compounds **2** and **6c** showed moderate activity; compounds **6b**, **8**, **10c**, **12**, and **14b** showed weak activity. However compound **15** exhibits very weak or no antioxidant activity (Table 5).

2.2.2.2.1. Structure–activity relationship (SAR) studies. The anti-microbial activity of the synthesized substituted imidazole derivatives is due to the presence of:

- Thiosemicarbazide fragment and quinoxaline nucleus (compound **2** and **8**).
- Thiazole ring (compound **6a**, **6b**, **7a**, **7b**, **8**, **9** and **10a**).
- Fluorinated arylidiene (compound **12**) arylidiene derivative with benzofuran substitute (compound **14b**).

Table 1
Characteristic data of the synthesized compounds.

Compd. No.	Mol. Formula (M. Wt)	Calcd. / Found C%	H%	N%	Mp. °C	Yield %
2	$C_{13}H_{15}N_5S$ (273.36)	57.12 / 57.41	5.53 / 5.64	25.62 / 25.43	245–6	72
6a	$C_{21}H_{19}N_5S$ (373.47)	67.53 / 67.45	5.13 / 5.23	18.75 / 18.66	261–2	61
6b	$C_{23}H_{19}N_5OS$ (413.49)	66.81 / 66.96	4.63 / 4.51	16.94 / 17.09	290–2	55
6c	$C_{24}H_{19}N_5O_2S$ (441.50)	65.29 / 65.38	4.34 / 4.45	15.86 / 15.90	218–9	56
7a	$C_{22}H_{21}N_7S$ (415.51)	63.59 / 63.78	5.09 / 5.21	23.60 / 23.73	237–9	68
7b	$C_{22}H_{20}ClN_7S$ (449.96)	58.72 / 58.67	4.48 / 5.03	21.79 / 21.06	186–7	66
8	$C_{21}H_{17}N_7S$ (399.47)	63.14 / 63.31	4.29 / 4.37	24.54 / 24.41	237–9	51
9	$C_{15}H_{15}N_5OS$ (313.38)	57.49 / 57.60	4.82 / 4.70	22.35 / 22.48	216–8	61
10a	$C_{22}H_{18}FN_5OS$ (419.47)	62.99 / 63.08	4.33 / 4.50	16.70 / 16.88	>300	A: 45 B: 56
10b	$C_{29}H_{23}N_7OS_2$ (549.67)	63.37 / 63.45	4.22 / 4.39	17.84 / 17.92	272–3	A: 41 B: 57
10c	$C_{33}H_{25}N_7O_2S$ (583.66)	67.91 / 67.98	4.32 / 4.47	16.80 / 16.71	>300	A: 48 B: 53
11	$C_{15}H_{15}N_5O$ (281.31)	64.04 / 64.21	5.37 / 5.49	24.90 / 24.99	255–6	82
12	$C_{22}H_{18}FN_5O$ (387.41)	68.21 / 68.37	4.68 / 4.53	18.08 / 18.25	201–2	86
14a	$C_{29}H_{23}N_7OS$ (517.60)	67.29 / 67.35	4.48 / 4.60	18.94 / 18.83	265–6	73
14b	$C_{33}H_{25}N_7O_2$ (551.60)	71.86 / 71.99	4.57 / 4.68	17.78 / 17.86	197–9	76
15	$C_{22}H_{19}N_5O_3$ (401.42)	65.83 / 65.71	4.77 / 4.63	17.45 / 17.63	253–4	68

Table 2
Antimicrobial activity chemical compounds against the pathological strains based on well diffusion assay.[a]

Chemical compound	*B. megaterium* ATCC9885	*B. subtilis* ATCC6633	*S. aureus* ATCC29213	*K. peneumoniae* ATCC13883	*P. aeroginosa* ATCC27953	*E. coli* ATCC25922	*Candida Albicans* NRRL Y-477	*A. niger*
2	++	+++	+	++	N.A.	N.A.	N.A.	N.A.
6a	N.A.	+++	+++	++	++	++	N.A.	N.A.
6b	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
6c	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
7a	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
7b	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
9	N.A..	+++	+++	+++	N.A.	N.A.	N.A.	N.A.
10a	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
10b	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
10c	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
11	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
12	++++	+++	++++	++	+++.	+++	N.A.	N.A.
14a	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
14b	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
15	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Ciprofloxacin	+++	++++	+++	++++	++++	+++	N.A.	N.A.
Ketoconazole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	++++	++++

[a] Antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm) as follows: N.A. (no activity) $\leq$ 4 mm; + (weak) = 5–9 mm; ++ (moderate) = 10–15 mm; +++ (strong) = 16–20 mm and ++++ (very strong) $\geq$ 21 mm. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

2.2.3. Antitumor activity using in vitro Ehrlich ascites assay

The newly synthesized compounds were screened for their antitumor activity. The viability of the cells used in control experiments exceeded 95%. Compound **6a** proved to have the highest cytotoxic activity (81.9%) followed by compounds **2**, **6c**, **7b** and **12**. Compound **15** showed no cytotoxic activity. The rest tested compounds showed very weak activity (Table 6).

2.2.3.1. Structure–activity relationship (SAR) studies. The antitumor activity of the synthesized substituted imidazole derivatives is due to the presence of:

- Thiosemicarbazide fragment (compound **2**), coumarin nucleus (**6c**) and 4-chlorobenzene azo substitute (**7b**).
- Fluorinated arylidiene (compounds **12**).

3. Experimental

3.1. Chemistry

All melting points were taken on Electrothermal IA 9000 series digital melting point apparatus. Elemental analytical data were carried from the microanalytical unit, National Research Centre, Dokki, Giza, Egypt. The IR spectra were recorded in potassium bromide disks on a Shimadzu CVT-04 spectrophotometer. The ^{1}H NMR spectra were recorded at 270 MHz on a Varian EM-360 spectrometer using TMS as an internal standard. Chemical shifts values (δ) are given in parts per million (ppm). The mass spectra were performed using mass Varian MAT CH-5 spectrometer at 70 eV. 4-Acetyl-5-methyl-2-phenylimdazole **1** [13], 1-(benzofuran-2-yl)-2-bromoethanone **3b** [18], 3-(2-bromoacetyl)-2*H*-chromen-2-one **3c** [19], hydrazonoyl chlorides **4a**, **b** [20], 2,3-dichloroquinoxaline **5** [21], 2-cyanoacetohydrazide [22], pyrazolyl aldehydes **13a** [23] and **13b** [24] were prepared according to the reported procedures.

*3.1.1. 2-(1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene) hydrazinecarbothioamide (**2**)*

To a solution of 4-acetyl-5-methyl-2-phenylimdazole **1** (2.00 g, 10 mmol) and thiosemicarbazide (0.91 g, 10 mmol) in absolute ethanol (20 mL), three drops of conc. HCl were added and the reaction mixture was refluxed for 5 h. The formed yellow to colorless precipitate was isolated by filtration, washed with ethanol, dried and recrystallized from dimethylformamide-water (DMF-H_2O) (3: 1 v/v). IR (KBr) ν_{max}/cm^{-1} 3335–3165 (NH_2, NH); ^{1}H NMR (DMSO-d_6) δ 1.31 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 7.61–8.12 (m, 5H, Ar-H), 8.56 (s, 2H, NH_2, D_2O-exchangeable), 10.51 (s, H, NH, D_2O-exchangeable), 12.91 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 273 (M^+, 50), 104 (100%).

*3.1.2. 2-{[1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene] hydrazono}-4-aryl-2,5-dihydrothiazoles (**6a–e**)*

A mixture of **2** (0.27 g, 1 mmol) and appropriate phenacyl bromide (1 mmol) in absolute ethanol (30 mL) was heated under reflux for 6 h. The formed solid was filtered off, washed with water, dried and recrystallized from EtOH/DMF to give the corresponding carbohydrazides **6a–e**.

*3.1.3. 2-{[1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene] hydrazono}-4-phenyl-2,5-dihydrothiazole (**6a**)*

^{1}H NMR (DMSO-d_6) δ 1.61 (s, 3H, CH_3), 2.62 (s, 3H, CH_3), 4.01 (s, 2H, CH_2), 7.41–7.92 (m, 10H, Ar-H), 13.81 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 373 (M^+, 28), 104 (100).

Table 3
Minimum inhibitory concentration (μg/mL) against the pathological strains based on two fold serial dilution technique.

Chemical compound	*B. megaterium* ATCC9885	*B. subtilis* ATCC6633	*S. aureus* ATCC29213	*K. peneumoniae* ATCC13883	*P. aeroginosa* ATCC27953	*E. coli* ATCC25922
2	–	87.5	–	–	–	–
6a	–	43.8	29.1	–	–	–
9	–	43.8	43.8	21.9	–	–
12	21.9	43.8	21.9	–	21.9	21.9
Ciproflxacine	21.9	10.9	21.9	10.9	10.9	21.9

Table 4
DPPH inhibition of chemical compounds.

Chemical compounds	DPPH IC_{50} (mg/mL)
2	6.25
6a	25
6b	25
6c	200
7a	25
7b	25
8	6.25
9	12.5
10a	6.25
10b	200
10c	200
11	100
12	200
14a	200
14b	200
15	100

*3.1.4. 4-(Benzofuran-2-yl)-2-{[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}-2,5-dihydrothiazole (**6b**)*

^{1}H NMR (DMSO-d_6) δ 2.30 (s, 3H, CH_3), 2.81 (s, 3H, CH_3), 4.10 (s, 2H, CH_2), 6.78 (s, 1H, benzofuryl-CH), 7.51–8.22 (m, 9H, Ar-H), 13.60 (s, H, NH, D_2O-exchangeable); ^{13}C NMR (DMSO-d_6) δ 12.06, 16.19, 39.86, 40.03, 40.19, 102.86, 107.41, 111.51, 121.96, 123.44, 123.82, 125.26, 127.95, 128.28, 128.96, 129.03, 129.82, 132.49, 142.45, 146.94, 152.27, 154.59, 170.35; MS *m*/*z* (%): 413 (M^+, 26), 184 (100).

*3.1.5. 3-{2-{[1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}-2,5-dihydrothiazol-4-yl}-2H-chromen-2-one (**6c**)*

^{1}H NMR (DMSO-d_6) δ 2.32 (s, 3H, CH_3), 2.88 (s, 3H, CH_3), 3.99 (s, 2H, CH_2), 7.42–8.28 (m, 10H, Ar-H), 13.45 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 441 (M^+, 30), 184 (100).

*3.1.6. 4-Methyl-2-{[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}-5-(2-arylhydrazono)-2,5-dihydrothiazoles (**7a**, **b**)*

A mixture of **2** (0.54 g, 2 mmol) and appropriate hydrazonoyl chlorides **4a**, **b** (2 mmol) in absolute ethanol (30 cm^3) containing triethylamine (0.2 g, 2 mmol) was heated under reflux for 3 h, then left cool. The formed precipitate was isolated by filtration, washed with ethanol, dried, and recrystallized from EtOH–DMF.

*3.1.7. 4-Methyl-2-{[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}-5-(2-phenylhydrazono)-2,5-dihydrothiazole (**7a**)*

^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, CH_3), 2.89 (s, 3H, CH_3), 3.22 (s, 2H, CH_3), 7.22–7.99 (m, 10H, Ar-H), 10.52 (s, H, NH, D_2O-exchangeable), 12.90(s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 415 (M^+, 45), 104 (100).

*3.1.8. 5-[2-(4-Chlorophenyl)hydrazono]-4-methyl-2-{[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}-2,5-dihydrothiazole (**7b**)*

^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, CH_3), 2.89 (s, 3H, CH_3), 3.22 (s, 2H, CH_3), 7.20–8.01 (m, 9H, Ar-H), 10.46 (s, H, NH, D_2O-exchangeable), 12.88 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 415 (M^+, 28%), 104 (100).

*3.1.9. 2-{[1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}-2,9a-dihydrothiazolo[5,4-b]quinoxaline (**8**)*

A mixture of thiosemicarbazide **2** (2.7, 10 mmol), 2,3-dichloroquinoxaline **7** (1.0 g, 10 mmol) in absolute ethanol (15 mL) was heated under refluxing conditions for 6 h. The formed solid product was filtered, dried and crystallized from ethanol to give **8**.

Table 5
Antioxidant assay for the prepared new compounds.

Compd. No.	ABTS[a] Inhibition (%)	Erythrocyte hemolysis (%)
L-ascorbic acid[b]	88.61	0.85
2	60.23	2.45
6a	79.35	2.75
6b	39.90	1.90
6c	61.65	2.50
7a	44.36	6.40
7b	40.72	1.70
8	37.40	1.10
10a	79.20	1.40
10b	76.20	3.45
10c	35.90	1.50
11	48.20	3.65
12	68.60	0.86
14a, **14b**	34.56	5.55
15	4.56	5.55

[a] 50 μL of (2 mM) of tested compounds in spectroscopic grade MeOH/phosphate buffer reaction mixture (1 mL, 1:1v/v).
[b] 50 μL of (2 Mm) of L-ascorbic acid was used in spectroscopic grade MeOH/phosphate buffer reaction mixture (1 mL, 1:1v/v).

^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, CH_3), 2.89 (s, 3H, CH_3), 3.64 (s, 1H, CH), 7.54–8.34 (m, 9H, Ar-H), 12.89 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 399 (M^+, 22%), 183 (100%).

*3.1.10. 2-{[1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}thiazolidin-4-one (**9**)*

A mixture of **2** (0.27 g, 1 mmol) and chloroacetic acid (0.1 g, 1 mmol) in glacial acetic acid (30 mL) containing anhydrous sodium acetate (0.33 g, 4 mmol) was heated under reflux for 6 h. The reaction mixture was cooled and the resulting precipitate was filtered off and recrystallized from ethanol to give **9**. IR (KBr) ν_{max}/cm^{-1} 3302–3107 (2NH); ^{1}H NMR (DMSO-d_6) δ 2.22 (s, 3H, CH_3), 3.21 (s, 3H, CH_3), 3.94 (s, 2H, CH_2), 7.34–7.89 (m, 5H, Ar-H), 9.09 (s, 1H, NH, D_2O-exchangeable), 11.99(s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 313 (M^+, 22%), 56 (100%).

*3.1.11. Synthesis of thiazolidin-4-ones **10a–c***

3.1.11.1. Method A. To a solution of 4-thiazolidinone **9** (0.313 g, 1 mmol) and appropriate aldehyde (1 mmol) in glacial acetic acid (20 mL), anhydrous sodium acetate (0.33 g, 4 mmol) was added and the reaction mixture was refluxed for 5 h then left to cool at room temperature. The formed solid was filtered off, dried and recrystallized from suitable solvent.

Table 6
Ehrlich in vitro assay for new synthesized compounds.[a]

Compd. No.	IC_{50} (μg/mL)
5-Florouracil	1.5
2	4.18
6a	19.5
6b	21.5
6c	125
7a	20.25
7b	20.25
8	4.25
10a	14.5
10b	8.25
10c	125.25
11	125.25
12	85.25
14a, **14b**	125.25
15	150.5

[a] Tested compounds were prepared (1 mg/mL) in 100 μL DMSO and complete to 1 mL using RPMI-1640 medium. 5-Florouracil (25 μg/mL) in 100 μL DMSO and complete to 1 mL using RPMI-1640 medium.

3.1.11.2. Method B. A mixture of **2** (0.27 g, 1 mmol), chloroacetic acid (0.1 g, 1 mmol) and appropriate aldehydes (1 mmol) in glacial acetic acid (20 mL) containing anhydrous sodium acetate (0.33 g, 4 mmol) was heated under reflux for 5 h. The reaction mixture was left to cool and the formed solid was filtered off, washed with water, dried and recrystallized from suitable solvent.

*3.1.12. 5-(4-Fluorobenzylidene)-2-{[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]hydrazono}thiazolidin-4-one (**10a**)*

IR (KBr) ν_{max}/cm^{-1} 3321–3049 (2NH), 1703(C=O); ^{1}H NMR (DMSO-d_6) δ 2.51 (s, 3H, CH_3), 2.93 (s, 3H, CH_3), 3.92 (s, 1H, NH, D_2O-exchangeable), 7.20–7.85 (m, 9H, Ar-H), 8.42 (s, 1H, CH = N–),11.99 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 419 (M^+, 39), 56 (100).

*3.1.13. 2-{[1-(5-Methyl-2-phenyl-1H-imidazol-4-yl)ethylidene] hydrazono}-5-((1-phenyl-3-(thiophen-2-yl)-1H-pyrazol-4-yl) methylene)thiazolidin-4-one (**10b**)*

IR (KBr) ν_{max}/cm^{-1} 3356–3044 (2NH), 1699 (C=O); ^{1}H NMR (DMSO-d_6) δ 2.48 (s, 3H, CH_3), 2.90 (s, 3H, CH_3), 3.95 (s, 1H, NH, D_2O-exchangeable), 7.21–7.84 (m, 8H, Ar-H), 8.62 (s, 1H, CH= N–),12.39 (s, H, NH, D_2O-exchangeable); ^{1}3C NMR (DMSO-d_6) δ 13.00, 16.03, 39.72, 39.88, 40.05, 40.21, 40.35, 96.86, 100, 116.65, 119.26, 127.67, 127.73, 128.32, 129.05, 130.24, 132.04, 134.77, 139.12, 146.09; MS *m*/*z* (%): 549 (M^+, 31), 56 (100).

*3.1.14. 5-{[3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl] methylene}-2-((1-(5-methyl-2-phenyl-1H-imidazol-4-yl) ethylidene)hydrazono)thiazolidin-4-one (**10c**)*

IR (KBr) ν_{max}/cm^{-1} 3336–3067 (2NH), 1699 (C=O); ^{1}H NMR (DMSO-d_6) δ 2.52 (s, 3H, CH_3), 2.93 (s, 3H, CH_3), 4.02 (s, 1H, NH, D_2O-exchangeable), 7.45–8.23 (m, 10H, Ar-H), 8.81 (s, 1H, CH=N–),11.98 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 583 (M^+, 29), 56 (100).

*3.1.15. 2-Cyano-N′-[1-(5-methyl-2-phenyl-1H-imidazol-4-yl) ethylidene]acetohydrazide (**11**)*

To a solution of 2-cyanoacetohydrazide (1.0 g, 10 mmol) in absolute ethanol (30 mL) 4-acetyl-5-methyl-2-phenylimidazole **1** (2.00 g, 10 mmol) was added. The reaction mixture was heated under reflux for 1 h then left to cool. The solid product formed upon pouring onto ice/water was collected by filtration.

IR (KBr) ν_{max}/cm^{-1} 3319 (NH); 2219 (CN), 1678 (C=O), ^{1}H NMR (DMSO-d_6) δ 2.32 (s, 3H, CH_3), 2.89 (s, 3H, CH_3), 4.08 (s, 2H, CH_2), 7.34–7.89 (m, 5H, Ar-H), 12.99 (s, H, NH, D_2O-exchangeable) 13.99 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 281 (M^+, 29), 82 (100).

*3.1.16. Synthesis of compounds **12**–**14***

3.1.16.1. General procedures. Equimolecular mixture of **11** (2.81 g, 0.01 mol) and appropriate aldehyde (0.01 mol), in anhydrous ethanol (20 mL) containing piperidine (0.50 mL) was heated under reflux for 3 h. The formed solid was collected by filtration.

*3.1.17. 2-Cyano-3-(4-fluorophenyl)-N′-[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]acrylohydrazide (**12**)*

IR (KBr) ν_{max}/cm^{-1} 3302–3073(2NH); 2221 (CN), 1683 (C=O), ^{1}H NMR (DMSO-d_6) δ 2.20 (s, 3H, CH_3), 2.78 (s, 3H, CH_3), 7.25–8.10 (m, 9H, Ar-H), 9.25 (s, 1H, CH=N), 12.98 (s, H, NH, D_2O-exchangeable) 14.24 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 372 (M^+, 31), 104 (100).

*3.1.18. 2-Cyano-N′-[1-(5-methyl-2-phenyl-1H-imidazol-4-yl) ethylidene]-3-[1-phenyl-3-(thiophen-2-yl)-1H-pyrazol-4-yl] acrylohydrazide (**14a**)*

IR (KBr) ν_{max}/cm^{-1} 3308–3103(2NH); 2221 (CN), 1681 (C=O), ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 3H, CH_3), 2.91 (s, 3H, CH_3), 7.22–8.46 (m, 13H, Ar-H), 9.31 (s, 1H, CH=N), 12.89 (s, H, NH, D_2O-exchangeable) 14.34 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 517 (M^+, 39), 104 (100).

*3.1.19. 3-[3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl]-2-cyano-N′-[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene] acrylohydrazide (**14b**)*

IR (KBr) ν_{max}/cm^{-1} 3319–3128 (2NH); 2220 (CN), 1689 (C=O), ^{1}H NMR (DMSO-d_6) δ 2.18 (s, 3H, CH_3), 2.87 (s, 3H, CH_3), 6.8 (s, 1H, benzofuryl-CH), 7.31–8.36 (m, 14H, Ar-H), 8.96 (s, 1H, CH=N), 12.35 (s, H, NH, D_2O-exchangeable) 13.96 (s, H, NH, D_2O-exchangeable); MS *m*/*z* (%): 551 (M^+, 23), 104 (100).

*3.1.20. 7-Hydroxy-2-imino-N′-[1-(5-methyl-2-phenyl-1H-imidazol-4-yl)ethylidene]-2H-chromene-3-carbohydrazide (**15**)*

IR (KBr) ν_{max}/cm^{-1} 3319–3128 (NH); 1661 (C=O), ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 3H, CH_3), 2.80 (s, 3H, CH_3), 6.68–7.90 (m, 8H, Ar-H), 8.41 (s, 1H, CH), 8.97 (s, H, NH, D_2O-exchangeable), 10.8 (s, 1H, OH, D_2O-exchangeable) 12.53 (s, H, NH, D_2O-exchangeable) 13.23 (s, H, NH, D_2O-exchangeable); ^{13}C NMR (DMSO-d_6) δ 12.44, 15.04, 39.87, 40.03, 40.20, 101.80, 111.33, 113.25, 116.30, 125.08, 128.43, 129.25, 130.85, 132.03, 135.58, 142.42, 143.52, 151.98, 155.85, 156.92, 158.37, 163.10.

3.2. Biological activities

3.2.1. Antimicrobial activity

Chemical compounds were individually tested against a panel of gram positive and negative bacterial pathogens. Antimicrobial tests were carried out by the agar well diffusion method [25] using 100 μL of suspension containing 1×10^8 CFU/mL of pathological tested bacteria, 1×10^6 CFU/mL of yeast and 1×10^4 spore/mL of fungi spread on nutrient agar (NA), Sabourand dextrose agar (SDA), and potato dextrose agar (PDA) medium respectively. After the media had cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 100 μL of tested compound solution prepared by dissolving 100 mg of the chemical compound in one mL of dimethyl sulfoxide (DMSO). The inculcated plates were then incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. Negative controls were prepared using DMSO employed for dissolving the tested compound. Ciprofloxacin (50 μg/mL) and Ketoconazole (50 μg/mL) were used as standard for antibacterial and antifungal activity respectively. After incubation time, antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms and compared with that of the standard. The observed zone of inhibition is presented in Table 1. Antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm) as follows: N.A. (no activity) ≤ 4 mm; +(weak) = 5–9 mm; ++(moderate) = 10–15 mm; +++(strong) = 16–20 mm and ++++(very strong) ≥ 21 mm. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

3.2.2. Minimal inhibitory concentration (MIC) measurement

The bacteriostatic activity of the active compounds (having inhibition zones (IZ) ≥ 16 mm) was then evaluated using the two fold serial dilution technique [26]. Two fold serial dilutions of the tested compounds solutions were prepared using the proper nutrient broth. The final concentration of the solutions was 250; 175; 87.5; 43.8 and 21.9 μg/mL. The tubes were then inoculated with the test organisms, grown in their suitable broth at 37 °C for 24 h for bacteria (about 1×10^8 CFU/mL), each 5 mL received 0.1 mL of the above inoculum and incubated at 37 °C for 24 h. The lowest concentration showing no growth was taken as the minimum inhibitory concentration (MIC).

3.2.3. Antioxidant activity

3.2.3.1. DPPH free radical and scavenging activity. The hydrogen atom or electron donation ability of the corresponding compounds was measured from the bleaching of purple colored of methanolic solution of DPPH. This spectrophotometric assay uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent [27,28]. Different concentrations of the chemical compounds were dissolved in methanol to obtain final concentration ranged from 6.25 to 200 mg/mL different concentrations were made to determine CI_{50} (concentration make 50% inhibition of DPPH color). Fifty microliters of various sample concentrations were added to 5 mL of 0.004% methanolic solution of DPPH. After a 60 min of incubation at dark, the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I %) was calculated as in Eq (2) :

$$I\% = \left(A_{blank} - A_{sample}\right) \Big/ (A_{blank}) \times 100 \quad (2)$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test sample.

3.2.3.2. Antioxidant activity screening assay for erythrocyte hemolysis. The blood was obtained from rats by cardiac puncture and collected in heparinized tubes. Erythrocytes were separated from plasma and the buffy coat was washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged at 2500 rev./min for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system [29]. A 10% suspension of erythrocytes in phosphate buffered saline pH 7.4 (PBS) was added to the same volume of 200 mM AAPH solution in PBS containing samples to be tested at different concentrations. The reaction mixture was shaken gently while being incubated at 37 °C for 2 h. The reaction mixture was then removed, diluted with eight volumes of PBS and centrifuged at 1500*g* for 10 min. The absorbance of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis, and the absorbance of the supernatant obtained after centrifugation was measured at 540 nm. The data percentage hemolysis was expressed as mean ± standard deviation. L-ascorbic acid was used as a positive control.

3.2.3.3. Antioxidant activity screening assay ABTS method. For each of the investigated compounds (2 mL) of ABTS solution (60 μM) was added to 3 mL MnO_2 solution (25 mg/mL), all prepared in (5 mL) aqueous phosphate buffer solution (pH 7, 0.1 M). The mixture was shaken, centrifuged, filtered and the absorbance of the resulting green-blue solution (ABTS radical solution) at λ 734 nm was adjusted to approx. ca. 0.5. Then, 50 μL of (2 mM) solution of the tested compound in spectroscopic grade MeOH/phosphate buffer (1:1) was added. The absorbance was measured and the reduction in color intensity was expressed as inhibition percentage. L-ascorbic acid was used as standard antioxidant (positive control). Blank sample was run without ABTS and using MeOH/phosphate buffer (1:1) instead of tested compounds. Negative control was run with ABTS and MeOH/phosphate buffer (1:1) only [30–32].

3.3. Cytotoxic activity

3.3.1. Ehrlich cells

Ehrlich cells (Ehrlich ascites Carcinoma, EAC) were derived from ascetic fluid from diseased mouse (the cells were purchased from National Cancer institute, Cairo, Egypt which is a certified institute by National Medical Research Ethics Committee). DNA (Calf Thymus type1), bleomycin sulfate, butylated hydroxyanisole (BHA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were obtained from sigma. 2,2′-azo-bis-(2-amidinopropane) dihydrochlorid (AAPH), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were purchased from Wako Co., USA.

3.3.2. Antitumor activity using Ehrlich ascites in vitro assay

Different concentrations of the tested compounds were prepared (100, 50 and 25 μl from 1 mg/mL in DMSO (<00.05%, v/v) and RPMI-1640 medium). Ehrlich cells (Ehrlich ascites Carcinoma, EAC) were derived from ascetic fluid from diseased mouse (purchased from National Cancer institute, Cairo, Egypt which is a certified institute by National Medical Research Ethics Committee). Ascites fluid from the peritoneal cavity of the diseased mouse (contains Ehrlich cells) was aseptically aspirated. The cells were grown partly floating and partly attached in a suspension culture in RPMI-1640 medium, supplemented with 10% fetal bovine serum. They were maintained at 37 °C in a humidified atmosphere with 5% CO_2 for 2 h. The viability of the cells determined by the microscopical examination using a hemocytometer and using trypan blue stain (stains only the dead cells) [33,34].

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