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Original article

Synthesis, antitumor, cytotoxic and antioxidant evaluation of some new pyrazolotriazines attached to antipyrine moiety

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ABSTRACT

Iminopropanehydrazonoyl cyanide **4** was achieved upon reaction of antipyrine diazonium salt **2** with 3-iminobutanenitrile (**3**) in EtOH/AcONa. 3-Aminopyrazole derivative **5** was obtained upon reaction of **4** with hydrazine hydrate. Diazodization of **5** afforded the diazonium salt **6** which coupled with active methylene compounds **7**–**10**, **19**, **20**, **25**, **29** and **32** in pyridine to give aryl hydrazone derivatives **11**–**14**, **21**, **22**, **26**, **30** and **33**, respectively. Refluxing of compounds **11**–**14**, **21**, **22**, **26** and **33** in acetic acid afforded the pyrazolotriazines **15**–**18**, **23**, **24**, **28** and **35**, respectively. The newly synthesized compounds were screened for their cytotoxic and antioxidant activities. The results showed clearly that compounds **4**, **5**, **13**, **22**, and **24** displayed promising *in vitro* anticancer activity against four different cell lines (HepG2, WI 38, VERO and MCF-7). Compounds **4** and **22** are the more potent antioxidant and anticancer agents. On the other hand, most of the compounds exhibited good cytotoxic activity toward (EAC).

1. Introduction

It is well known that nitriles are widely used as intermediates for a large number of heterocyclic compounds. Aminopyrazole compounds can be readily obtained by the reaction of nitrile derivatives with hydrazine hydrate [1–4].

Also, aminopyrazole is often used for construction of pyrazolotriazine heterocyclic systems *via* diazodization and diazocoupling with active methylene moieties [5–7]. Antipyrine or phenazone derivatives are well known compounds used mainly as analgesic and antipyretic drugs [8]. One of the best known antipyrine derivatives is 4-aminoantipyrine which is used for the protection against oxidative stress as well as prophylactic of some diseases including cancer and these are important directions in medicine and biochemistry [9,10]. Reactive oxygen species (ROS), including free radicals led to a decrease in the antioxidant capacity and may generate other reactive species that damage the living cell. Oxidative stress may arise in a biological system after an increased exposure to oxidants, so the antioxidants play a major role in protecting biological systems against such threats. Different types of antioxidants (vitamins C and E, glutathione, lipoic acid and butylated phenols, etc.) have been widely used in different fields of industry and medicine as compounds that interrupt radical-chain oxidation processes, causing thus a high scientific interest [11,12]. Antipyrine derivatives are strong inhibitors of cycloxygenase isoenzymes, platelet tromboxane and prostanoids synthesis [8,13]. The biological activity of these compounds has also been attributed to its scavenging activity against reactive oxygen and nitrogen species, as well as to the inhibition of neutrophil's oxidative burst. However, besides its well recognized benefits, antipyrine derivatives have been associated with potential adverse effects characterized by leukopenia, most commonly of neutrophils, causing neutropenia in the circulating blood (agranulocytosis). It is worth to mention that there are studies demonstrating that this adverse effect might be exaggerated [14,15].

Moreover, several pyrazole ring systems are associated with antifungal, antitubercular, antibacterial, antiviral anticancer and antioxidant activities [16–18] as well as the biological activities of pyrazolotriazine ring systems are well documented [19–21]. It has been used as adenine analogs, antagonists, antischistosomal, antitumor and antibacterial agents [22–27]. Therefore, it is a real challenge to combine the above mentioned boilable rings together in a molecular framework to see the additive effect of these rings toward the antioxidant and antitumor activities.

2. Result and discussion

The synthetic strategies adopted to obtain the corresponding pyrazolotriazines are depicted in Schemes 1–4. The starting

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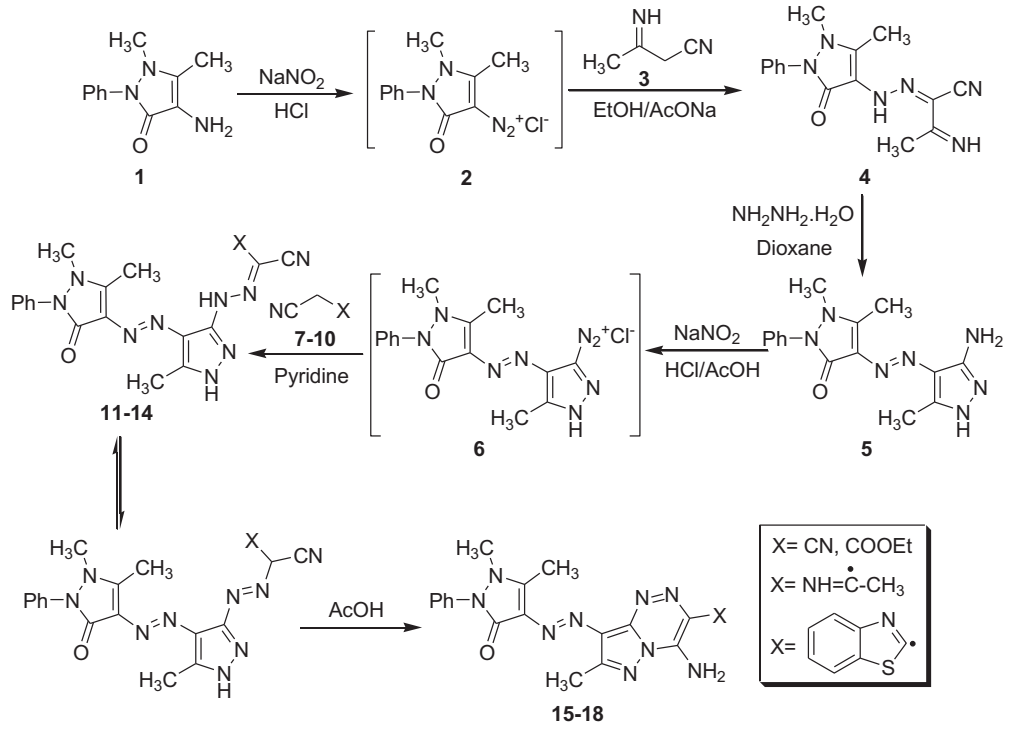
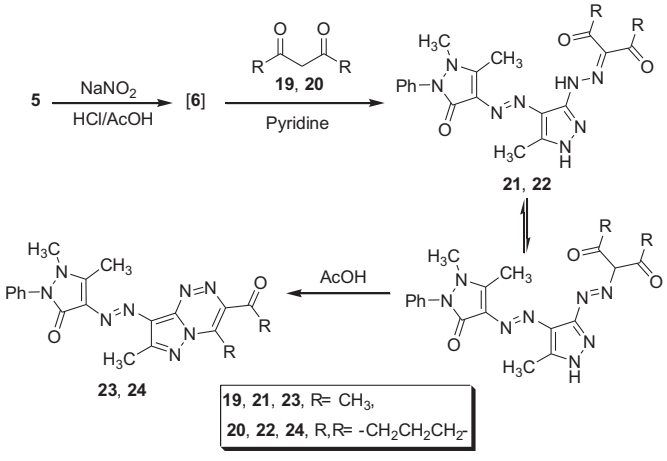


Scheme 1. Synthetic route for 3-substituted-7-methylpyrazolo[5,1-*c*][1,2,4]triazines **15**–**18**.

material, 4-((3-amino-5-methyl-1*H*-pyrazol-4-yl)diazenyl)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**5**) was prepared by coupling of 1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazole-4-diazonium chloride (**2**) with 3-iminobutanenitrile (**3**) [28] in ethanol containing sodium acetate followed by heating the formed *N*′-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-2-iminopropane hydrazonoyl cyanide (**4**) under reflux with hydrazine hydrate in dioxane. Diazotization of aminopyrazole **5** with sodium nitrite in a mixture of acetic acid and hydrochloric acid led to the corresponding diazonium salt [intermediate] **6** which coupled with malononitrile (**7**), ethyl cyanoacetate (**8**), 3-iminobutanenitrile (**9**) or 2-(benzo[*d*]thiazol-2-yl)acetonitrile (**10**) [29] in pyridine to give the corresponding hydrazones **11**–**14**, respectively. Cyclizations of compounds **11**–**14** afforded the desired pyrazolotriazines **15**–**18** under the influence of acetic acid (Scheme 1).



Scheme 2. Synthesis of 3,4-disubstituted-7-methylpyrazolo[5,1-*c*][1,2,4]triazines **23** and **24**.

In similar manner, the diazonium salt **6** reacted with acetylacetone (**19**) or cyclohexane-1,3-dione (**20**) in pyridine to afford products that may be formulated as hydrazone derivatives **21** and **22**, respectively based on both elemental analyses and spectral data. Cyclizations of the resulted hydrazones **21** and **22** under acidic condition gave the desired pyrazolotriazines **23** and **24**, respectively (Scheme 2).

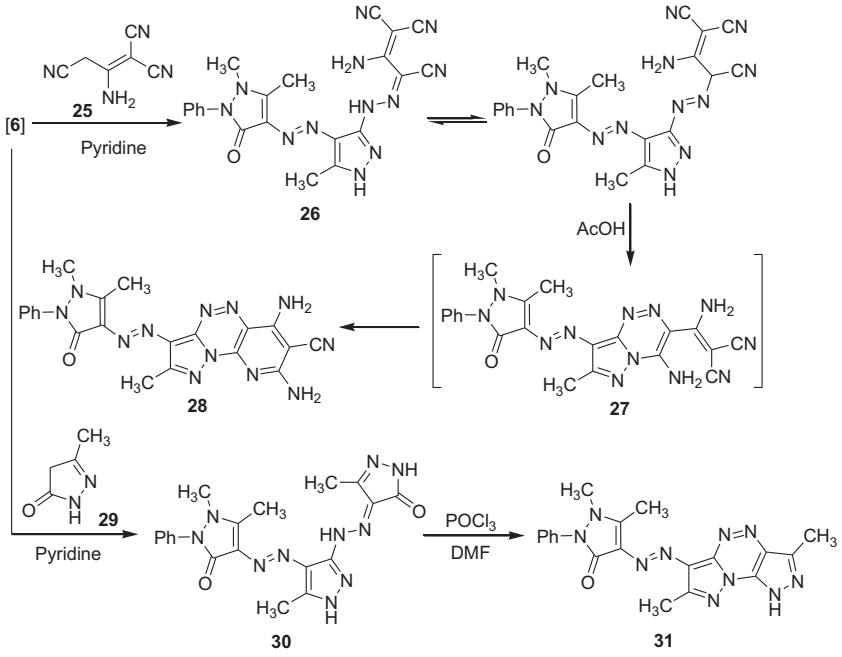
The synthetic potency of **6** was investigated to develop a facile and convenient route to polysubstituted pyrazolotriazine derivatives of expected biological activity [30]. Thus, coupling of compound **6** with 2-aminoprop-1-ene-1,1,3-tricarbonitrile (**25**) [31] in pyridine afforded hydrazone derivative **26** which gave pyrazolopyridotriazine derivative **28** upon refluxing in acetic acid through the intermediate **27**. Furthermore, we have investigated the reactivity of **6** with 3-methyl-1*H*-pyrazol-5(4*H*)-one (**29**) [32] to synthesize dipyrazolo[5,1-*c*:3′,4′-*e*][1,2,4]triazine ring system **31**. Thus, coupling of diazonium salt **6** with compound **29** in pyridine furnished the hydrazone derivative **30** which cyclized in $POCl_3$/DMF to afford the target compound **31** (Scheme 3).

Treatment of **6** with 1-phenyl-2-thiocyanatoethanone (**32**) in pyridine gave hydrazone derivative **33**. The preparation of compound **34** through cyclization of **33** in acetic acid was failed and we obtain the iminothiadiazole **35** (Scheme 4). Assignment of the new synthesized compounds was based on elemental analyses and spectral data (IR, ^{1}H NMR and mass spectra) (*C.f.* Experimental Part).

2.1. Biological studies

2.1.1. Antitumor activity

2.1.1.1. Effect of drugs on the viability of Ehrlich ascites cells (EAC) in vitro. Twenty antipyrine derivatives were tested for cytotoxicity against a well known established model EAC *in vitro* [33]. Results for the ED_{100}, ED_{50}, ED_{25} and IC_{50} values of the active compounds are summarized in Table 1. The data showed clearly that most of compounds have good activities. Thus, it would appear that the

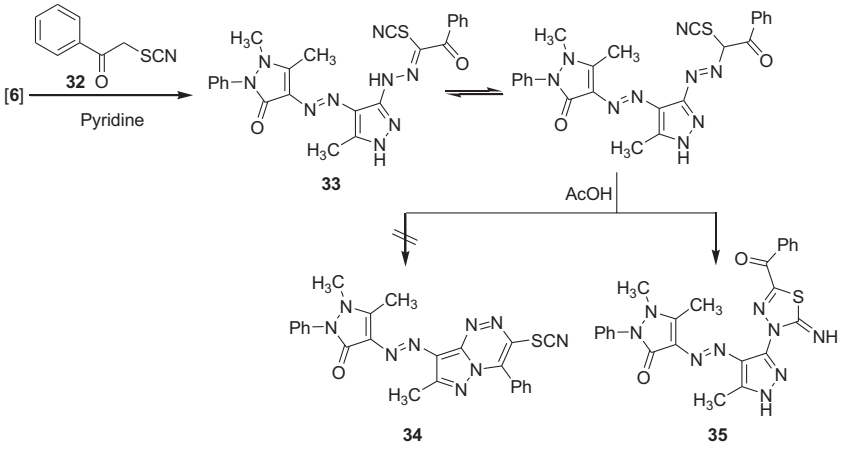


Scheme 3. Synthesis of pyrazolo[5,1-*c*]pyrido[2,3-*e*][1,2,4]triazine **28** and dipyrazolo[5,1-*c*:3′,4′-*e*][1,2,4]triazine **31**.

presence of a basic skeleton of antipyrine, pyrazole or triazine moieties are necessary for the good results of cytotoxic activity against (EAC). By comparing the results obtained of the investigated compounds to their structures the following structure activity relationships (SAR's) were postulated: (i) Antipyrine derivative **4** is less potent than pyrazole **5** which may be attributed to the conversion of iminobutanenitrile moiety into aminopyrazole. (ii) Most of the hydrazone derivatives are less potent than the corresponding pyrazolotriazine which may be attributable to the cyclization of hydrazone into triazine moiety. (iii) Compounds **26** and **28** showed very high cytotoxic activity ($IC_{50} = 77.1$ and 78, respectively) which may be due to presence of hydrazono-malononitrile dimer and triazine moiety, respectively. (iv) Compound **24** is less potent than **23** which may be due to presence of cyclohexane moiety.

2.1.2. Cytotoxicity and in vitro anticancer evaluation

Out of the newly synthesized compounds, twenty analogs were selected to be evaluated for their *in vitro* anticancer effect *via* the standard MTT method [34–36], against a panel of four human tumor cell lines namely; heptacelluar carcinoma (HepG2), lung fibroblasts (WI 38), kidney of a normal adult African green monkey (VERO) and breast cancer (MCF-7). The cell lines were obtained from ATCC *via* the Holding company for biological products and vaccines (VACSERA) (Cairo, Egypt). 5-Fluorouracil (5-FU) was used as a standard anticancer drug. MTT assay is a standard colorimetric assay for measuring cell growth. It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. In brief, yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenases of living cells. A suitable solvent is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength. When the amount of purple formazan produced by cells treated with an agent is compared with that produced by unreacted control cells, the effectiveness of the agent in causing death of cells can deduced through the production of



Scheme 4. Synthesis of 4-((3-(5-benzoyl-2-imino-1,3,4-thiadiazol-3(2*H*)-yl)-5-methyl-1*H*-pyrazol-4-yl)diazenyl)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**35**).

Table 1
In vitro antitumor assay for 5-fluorouracil and the investigated compounds.

Compound no.	IC_{50} μg	ED_{100} μg	ED_{50} μg	ED_{25} μg
5-FU	97.3	68	38.6	32.26
4	100	97.2	84.3	0.46
5	97	79.3	58	1.71
11	94.3	64	48	27.85
12	94.9	68.7	49.8	22.53
13	97.5	80	60.1	1.30
14	95	70.8	56.4	11.17
15	91.9	62	42.8	34.26
16	94	65.3	47	28.02
17	95.5	70.1	55	14.38
18	93.8	64	49	26.63
21	95	70	54.1	15.59
22	98.8	80	63.3	0.88
23	96.7	73.9	55.5	11.01
24	99	91.4	80.2	0.67
26	77.1	38	21	64.48
28	78	40.6	22	62.53
30	93	63.3	45.5	26.63
31	92.1	61	43	34.69
33	94.3	65.2	48.1	26.92
35	90.7	60.5	38	39.36

Where, ED_{100}, ED_{50}, and ED_{25} are the effective doses at 25, 50, and 100 μg, respectively, of the compounds used. The dead % refers to the % of the dead tumor cells and 5-FU is 5-fluorouracil as a well known cytotoxic agent.

a dose response curve. The obtained results (Table 2) revealed that compounds **4**, **5**, **13**, **22** and **24** exhibited variable degrees of inhibitory activity toward the four tested human tumor cell lines. As for activity against HepG2 cell line, the very highest cytotoxic activity was displayed by compound **4** which showed the percentage viability IC_{50} at 8.3 mg/mL, whereas, the highest cytotoxic activity was displayed by compound **22** which showed the percentage viability IC_{50} at 16.2 mg/mL and weak inhibitory activity was also demonstrated by compounds **5**, **13** and **24**. The WI 38 cell line showed highest sensitivity toward compounds **4** and **22** which have IC_{50} at 13.8 and 19.8 mg/mL, respectively, whereas, compounds **5**, **13** and **24** displayed weak cytotoxic activities. On the other hand, compound **4** showed very highest cytotoxic activity IC_{50} at 10.0 mg/mL toward VERO cell line, compound **22** showed the highest cytotoxic activity for the percentage viability IC_{50} at 24.8 mg/mL against VERO cell line. The remaining two active compounds **5** and **24** showed moderate activity against the same cell line at IC_{50} 80.3 and 61.1 mg/mL, respectively. Furthermore, interpretation of the results revealed that compounds **4**, **5**, **13**, **22** and **24** vary from very strong to moderate anticancer activity against MCF-7 cell line with percentage inhibition IC_{50} at 18.1, 29.9, 84.7, 69 and 68.2 mg/mL, respectively.

Table 2
Cytotoxicity (IC_{50}) of tested compounds on different cell lines.

Compound no.	IC_{50} (μg/mL)*			
	HepG2	WI 38	VERO	MCF-7
5-FU	9.1	4.0	6.4	2.5
4	8.3	13.8	10.0	15.1
5	79.1	73.1	80.3	84.7
11	182.6	197.2	208.7	221.2
12	153.2	169.2	179.2	150.0
13	70.8	70.2	734.6	69.0
14	178.9	169.3	180.8	164.1
15	254.1	377.0	301.1	451.7
16	268.4	367.1	284.5	420.2
17	138.7	175.1	164.2	151.8
18	225.2	198.7	243.6	185.7
21	174.0	188.6	199.2	214.3
22	16.2	19.8	24.8	29.9
23	39.3	40.3	33.1	30.6
24	55.3	53.0	61.1	68.2
26	147.9	175.9	212.3	191.8
28	203.4	303.3	220.2	418.8
30	202.0	299.7	213.7	393.2
31	209.8	310.8	276.5	411.3
33	95.3	92.8	96.8	100.7
35	186.3	171.3	210.3	209.6

IC_{50}, (μg/mL): 1–10 (very strong), 11–25 (strong), 26–50 (moderate), 51–100 (weak), 100–200 (very weak), above 200 (non-cytotoxic).

2.1.3. Antioxidant activity assay

The antioxidant activities of twenty pyrazolotriazine compounds were evaluated same as reported by Lissi et al. [37]. The data showed clearly that, compounds **4** has good activity, while compound **22** and **24** exhibited moderate activities. On the other hand, the other compounds showed weak activities (Table 3 and Fig. 1). Thus, it would appear that introducing of aminopyrazole moiety enhances the antioxidant properties of aminoantipyrine ring system.

2.1.4. Bleomycin-dependent DNA damage

The bleomycine is a family of glycopeptide antibiotics that is used routinely as antitumor agents. The bleomycin assay has been adopted for assessing the pro-oxidant effects of food antioxidants. The antitumor antibiotic bleomycin binds iron ions and DNA. The bleomycine iron complex degrades DNA that, upon heating with thiobarbituric acid (TBA), yields a pink chromogen. Upon the addition of suitable reducing agents antioxidants compete with DNA and diminish chromogen formation [38]. To show the mechanism of action of our potent compounds **4**, **5**, **13**, **22** and **24**, their protective activity against DNA damage induced by the bleomycine iron complex were examined. The results in Table 4 showed that compounds **4**, **22**, **24** and **33** exhibited high protection against DNA damage induced by the bleomycine iron complex, thus diminishing chromogen formation between the damaged DNA and TBA molecules.

2.2. Structure activity relationship's (SAR's)

By comparing the experimental cytotoxicity of the compounds reported in this study to their structures, the following structure activity relationship's (SAR's) were postulated. (i) The presence of a basic skeleton of antipyrine moiety is necessary for the broad

Table 3
Antioxidant activity assay (ABTS) of some new pyrazolotriazine compounds.

Entry	Compound no.	% Inhibition
1	Control of ABTS	0.0
2	Ascorbic acid	92.30
3	**4**	80.93
4	**5**	28.38
5	**11**	17.16
6	**12**	21.61
7	**13**	28.60
8	**14**	19.27
9	**15**	15.88
10	**16**	17.58
11	**17**	24.36
12	**18**	17.16
13	**21**	25.84
14	**22**	40.04
15	**23**	27.54
16	**24**	52.54
17	**26**	9.86
18	**28**	8.28
19	**30**	16.10
20	**31**	16.10
22	**33**	17.58
23	**35**	15.67

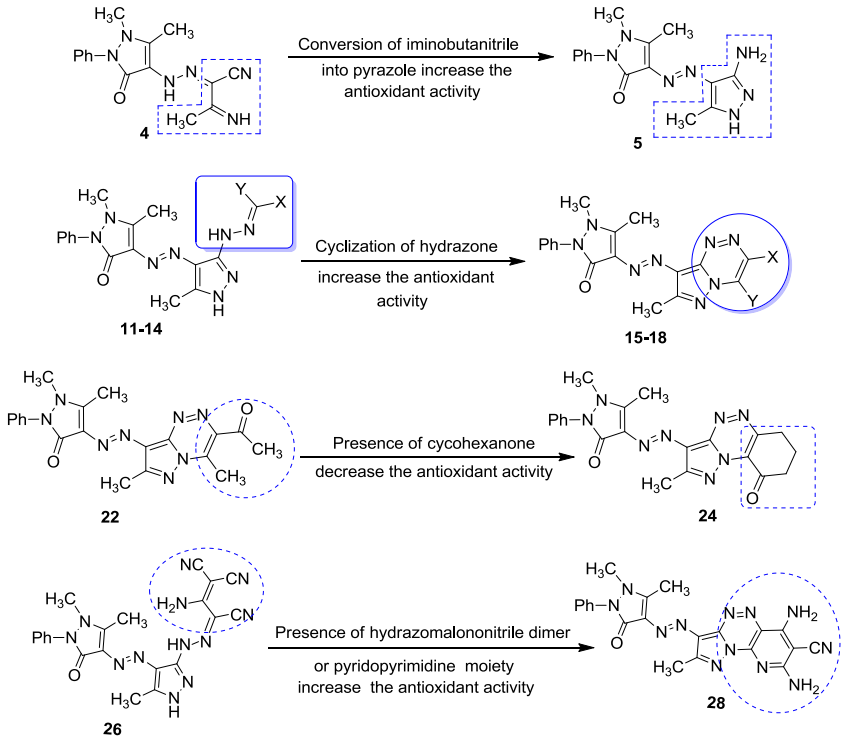


Fig. 1. Structure activity relationships (SAR's) of the more potent antitumor and antioxidant compounds.

spectrum of cytotoxic activity toward different cell lines (HepG2, WI 38, VERO and MCF-7). (ii) Antipyrine derivative **4** is more potent than 5-florouracil toward HepG2 which may be attributed to the replacement of pyrimidine with the antipyrine moiety and the presence of iminobutanenitrile moiety. (iii) Compound **5** is less potent than compound **4** which may be attributed to the conversion of the iminobutanenitrile into pyrazole moiety. (iv) Most of the hydrazone derivatives are more potent than pyrazolotriazine compounds which may be attributable to the presence of hydrazone moiety. (v) Compound **24** is less potent than **23** which may be due to cyclization of hydrazone moiety into pyrimidine ring system. (vi) Cyclization of thioamide **9** to 2,3-dihydrothiazole derivative **7a** enhances the antitumor activity against different cell lines.

3. Conclusion

The objective of the present study was to synthesize and evaluate the antitumor and antioxidant activity of some novel pyrazolotriazines with the hope of discovering new structure leads serving as antitumor and antioxidant gents. The data showed clearly that compounds **4**, **5**, **13**, **22** and **24** displayed promising *in vitro* antitumor (against four cell lines) and antioxidant activities, while compounds **4** and **22** exhibited broad spectrum of antitumor and antioxidant activities. Most of the tested compounds exhibited good cytotoxic activity toward (EAC), so, there are relationships between the cancer and oxidant results [9,10].

Table 4
Results of bleomycin-dependent DNA damage assay on some selected compounds.

Compound no.	Absorbance of samples
Ascorbic acid	0.098
4	0.063
5	0.124
13	0.117
22	0.080
24	0.089
33	0.101

4. Experimental

4.1. General

All melting points are in degree centigrade (uncorrected) and were determined on Gallenkamp electric melting point apparatus. Elemental analyses (C, H and N) were carried out at the Microanalytical Center of Cairo Univ., Giza, Egypt. The IR spectra were recorded (KBr) on a Mattson 5000 FTIR Spectrophotometer at the Microanalytical Center (Faculty of Science; Mansoura University). The ^{1}H NMR spectra were measured on a Varian Spectrophotometer at 300 MHz, using TMS as an internal reference and DMSO-d_6 as solvent at the Microanalytical Center (Faculty of Science, Cairo University). The mass spectra were recorded on Shimadzu Qp-2010 plus at the Microanalytical Center; (Faculty of Science; Cairo University). Biological activity was carried in Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

4.1.1. Synthesis of N′-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-2-iminopropanehydrazonoyl cyanide (4)

A well stirred solution of 4-aminoantipyrine (**1**) (1.02 g, 5 mmol) in conc. HCl (3 mL) and 2 mL H_2O was cooled in ice bath and diazotized with the solution of $NaNO_2$ (0.35 g, 5.1 mmol in 5 mL H_2O). The cold diazonium solution was added slowly to a well stirred solution of **3** (0.41 g, 5 mmol) in ethanol (20 mL) containing sodium acetate (1.64 g, 20 mmol). The reaction mixture was stirred for another 2 h. The crude product was filtered off, dried well and recrystallized from ethanol/benzene mixture to give **4**. Yield (78%); orange crystals; m.p. 218–220 °C; IR (KBr): ν/cm^{-1} = 3299, 3203 (2NH), 2190 (CN), 1646 (CO), 1612 (C=N), 1490 (N=N); ^{1}H NMR

(DMSO-d_6) δ (ppm): 2.23 (s, 3H, CH_3, pyrazole), 2.29 (s, 3H, CH_3–C═N), 3.22 (s, 3H, CH_3–N), 7.32–7.54 (m, 5H, Ar–H), 7.64 (br, 1H, C═NH), 7.97 (br, 1H, NH, hydrazo); MS (EI, 70 eV) m/z (%) = 298 ($M^+ + 2$, 2.1), 297 ($M^+ + 1$, 8.9), 296 (M^+, 1.1), 202 (9.2), 91 (10.8), 83 (43.0), 77 (10.3), 56 (100.0), 55 (8.1). Anal. Calcd. for $C_{15}H_{16}N_6O$ (296.33): C, 60.80; H, 5.44; N, 28.36%. Found: C, 60.86; H, 5.49; N, 28.46%.

4.1.2. Synthesis of 4-((3-amino-5-methyl-1H-pyrazol-4-yl) diazenyl)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**5**)

To a solution of **4** (1.48 g, 5 mmol) in dioxane (20 mL) and hydrazine hydrate (0.25 g; 98%, 5 mmol) was added. After refluxing for 4 h, the reaction mixture was cooled then the formed precipitate was filtered off, dried and recrystallized from DMF/EtOH to give **5**. Yield (90%); orange powder; m.p. 288–290 °C; IR (KBr): ν/cm^{-1} = 3451, 3262, 3201 (NH_2, NH), 1641 (CO), 1610 (C═N), 1484 (N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.29 (s, 3H, CH_3-antipyrine), 2.33 (s, 3H, CH_3-pyrazole), 3.19 (s, 3H, CH_3-antipyrine), 6.08 (br, 2H, NH_2), 7.35–7.56 (m, 6H, Ar–H, NH-pyrazole); MS (EI, 70 eV) m/z (%) = 312 ($M^+ + 1$, 7.1), 311 (M^+, 37.0), 296 (2.1), 119 (5.5), 77 (9.2), 56 (100.0). Anal. Calcd. for $C_{15}H_{17}N_7O$ (311.34): C, 57.87; H, 5.50; N, 31.49%. Found: C, 57.96; H, 5.57; N, 31.53%.

4.1.3. Synthesis of 4-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yllazo)-5-methyl-pyrazol-3-yl-hydrazones **11**–**14**, **21**, **22**, **26**, **30** and **33**

General procedure: A well stirred solution of **5** (1.56 g, 5 mmol) in a mixture of acetic acid (10 mL) and conc. HCl (3 mL) was cooled in an ice bath and then a solution of $NaNO_2$ (0.35 g, 5.1 mmol in 5 mL H_2O) was added dropwise to it. The above cooled diazonium solution was added slowly to a well stirred solution of active methylene compounds namely; malononitrile (**7**) (0.33 g, 5 mmol) or ethyl-2-cyanoacetate (**8**) (0.57 g, 5 mmol) or 3-iminobutanenitrile (**9**) (0.41 g, 5 mmol) or 2-(benzo[*d*]thiazol-2-yl)acetonitrile (**10**) (0.87 g, 5 mmol) or acetylacetone (**19**) (0.5 g, 5 mmol) or cyclohexane-1,3-dione (**20**) (0.56 g, 5 mmol) or 2-iminopropane-1,1,3-tricarbonitrile (**25**) (0.66 g, 5 mmol) or 3-methyl-1*H*-pyrazol-5(4*H*)-one (**29**) (0.49 g, 5 mmol) or 1-phenyl-2-thiocyanatoethanone (**32**) (0.89 g, 5 mmol) in pyridine (15 mL). The reaction mixture was stirred for 2 h. The crude product was filtered off, dried well and crystallized from the appropriate solvent to give compounds **11**–**14**, **21**, **22**, **26**, **30** and **33**, respectively.

4.1.3.1. (4-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)carbonohydrazonoyl dicyanide (**11**).

Yield (67.0%); orange crystals; m.p. 226–228 °C; IR (KBr): ν/cm^{-1} = 3442, 3423, 3410 (NH_2, 2NH), 2220 (CN), 1646 (CO), 1496 (N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.49 (s, 3H, CH_3-antipyrine), 2.66 (s, 3H, CH_3-pyrazole), 3.37 (s, 3H, CH_3–N), 7.36–7.59 (m, 6H, NH-pyrazole, Ar–H), 9.40 (br, 1H, NH-hydrazone); MS (EI, 70 eV) m/z (%) = 389 ($M^+ + 1$, 3.7), 388 (M^+, 6.3), 374 (4.8), 311 (9.1), 296 (4.6), 281 (4.3), 214 (24.4), 202 (14.1), 189 (39.5), 174 (29.8), 148 (24.6), 132 (2.6), 121 (40.2), 93 (45.5), 77 (45.0), 56 (100.0), 53 (38.9). Anal. Calcd. for $C_{18}H_{16}N_{10}O$ (388.39): C, 55.66; H, 4.15; N, 36.06%. Found: C, 55.71; H, 4.26; N, 36.18%.

4.1.3.2. Ethyl 2-cyano-2-(2-(4-((E)-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl) hydrazono)acetate (**12**).

Yield (77.0%); orange crystals; m.p. 175–177 °C; IR (KBr): ν/cm^{-1} = 3222 (br, 2NH), 2206 (CN), 1718, 1668 (2CO), 1448 (2N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 1.31 (t, 3H, CH_3, J = 7.2 Hz), 2.43 (s, 3H, CH_3), 2.66 (s, 3H, CH_3-pyrazole), 3.30 (s, 3H, CH_3–N), 4.28 (q, 2H, OCH_3, J = 7.2 Hz), 7.43–7.95 (m, 6H, Ar–H, NH-pyrazole), 12.5 (br, 1H, NH-hydrazone); MS (EI, 70 eV) m/z (%) = 436 ($M^+ + 1$, 4.2), 435 (M^+, 11.7), 363 (4.5), 236 (6.3), 214 (5.0), 202 (6.2), 149 (12.3), 137 (4.2), 119 (18.0), 109 (7.0), 91 (26.9), 77 (23.1), 56 (100.0), 55 (17.0). Anal. Calcd. for $C_{20}H_{21}N_9O_3$ (435.44): C, 55.17; H, 4.86; N, 28.95%. Found: C, 55.24; H, 4.93; N, 29.03%.

4.1.3.3. N′-(4-((E)-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)-2-iminopropanehydrazonoyl cyanide (**13**).

Yield (82.0%); black crystals; m.p. <300 °C; IR (KBr): ν/cm^{-1} = 3423, 3255, 3224 (3NH), 2200 (CN), 1737 (br, CO, C═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.3 (s, 3H, CH_3, antipyrine), 2.49 (s, 3H, CH_3–C═N), 2.66 (s, 3H, CH_3, pyrazole), 3.3 (s, 3H, CH_3–N), 7.36–7.58 (m, 7H, 2NH, Ar–H), 12.15 (br, 1H, NH, hydrazone); MS (EI, 70 eV) m/z (%) = 405 ($M^+ + 1$, 1.1), 404 (M^+, 0.8), 387 (29.7), 203 (7.5), 188 (28.7), 173 (3.3), 147 (18.2), 138 (3.3), 120 (25.7), 109 (4.2), 91 (17.4), 77 (17.9), 56 (100.0), 52 (9.3). Anal. Calcd. for $C_{19}H_{20}N_{10}O$ (404.43): C, 56.43; H, 4.98; N, 34.63%. Found: C, 56.47; H, 5.02; N, 34.67%.

4.1.3.4. N′-(4-((E)-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)benzo[d]thiazole-2-carbohydrazonoyl cyanide (**14**).

Yield (65.0%); yellow crystals; m.p. 208–210 °C; IR (KBr): ν/cm^{-1} = 3426, 3208 (2NH), 2250 (CN), 1648 (CO), 1515 (N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.41 (s, 3H, CH_3, antipyrine), 2.69 (s, 3H, CH_3, pyrazole), 3.27 (s, 3H, CH_3–N), 7.35–8.27 (m, 10H, Ar–H, NH, pyrazole), 12.88 (br, s, 1H, NH-hydrazone); MS (EI, 70 eV) m/z (%) = 498 ($M^+ + 2$, 7.2), 497 ($M^+ + 1$, 4.3), 496 (M^+, 10.4), 482 (6.3), 297 (16.2), 282 (10.7), 255 (14.8), 229 (10.5), 174 (23.1), 161 (54.1), 146 (15.9), 135 (13.5), 119 (30.2), 109 (27.7), 91 (33.3), 77 (35.9), 56 (100.0), 54 (19.0). Anal. Calcd. for $C_{24}H_{20}N_{10}OS$ (496.55): C, 58.05; H, 4.06; N, 28.21%. Found: C, 58.10; H, 4.14; N, 28.26%.

4.1.3.5. 3-(2-(4-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)hydrazono)pentane-2,4-dione (**21**).

Yield (76.0%); dark brown crystals; m.p. > 300 °C; IR (KBr): ν/cm^{-1} = 3405, 3233 (2NH), 1680, 1637 (3CO), 1494 (N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.36 (s, 3H, CO–CH_3), 2.37 (s, 3H, CH_3–CO), 2.44 (s, 3H, CH_3-antipyrine), 2.60 (s, 3H, CH_3-pyrazole), 3.28 (s, 3H, N–CH_3), 7.39-7.58 (m, 6H, Ar–H, NH-pyrazole), 11.2 (br, NH, NH-hydrazone); MS (EI, 70 eV) m/z (%) = 406 ($M^+ - H_2O$, 6.5), 405 (10.0), 404 (36.1), 205 (12.4), 91 (17.7), 83 (17.0), 77 (14.3), 67 (24.0), 56 (100.0), 51 (6.5). Anal. Calcd. for $C_{20}H_{22}N_8O_3$ (422.44): C, 56.86; H, 5.25; N, 26.53%. Found: C, 56.96; H, 5.36; N, 26.59%.

4.1.3.6. 2-(2-(4-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)hydrazono)cyclohexane-1,3-dione (**22**).

Yield (64.0%); dark brown crystals; m.p. 223–225 °C; IR (KBr): ν/cm^{-1} = 3386, 3206 (2NH), 1675, 1632 (3C═O), 1496 (N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 1.85–1.93 (m, 2H, CH_2, cyclohexane) 2.4–2.7 (m, 10H, $2CH_3$, $2CH_2$, cyclohexanone), 2.74 (s, 1H, CH, cyclohexanone), 3.21 (s, 3H, CH_3–N), 7.36–7.60 (m, 5H, Ar–H), 9.00 (s, 1H, NH); MS (EI, 70 eV) m/z (%) = 434 (M^+, 65.0), 279 (79.0), 239 (77.1), 227 (85.4), 210 (95.5), 207 (31.9), 189 (65.0), 174 (8.9), 160 (77.7), 144 (74.0), 134 (17.8), 124 (13.4), 107 (19.1), 71 (20.4), 59.95 (100.0). Anal. Calcd. for $C_{21}H_{22}N_8O_3$ (434.45): C, 58.06; H, 5.10; N, 25.79%. Found: C, 58.12; H, 5.14; N, 25.83%.

4.1.3.7. 2-Amino-3,3-dicyano-N′-(4-((E)-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)acrylohydrazonoyl cyanide (**26**).

Yield (76.0%); dark red crystals; m.p. >300 °C; IR (KBr): ν/cm^{-1} = 3388, 3299, 3197 (3NH), 2198 (CN), 1650 (CO), 1623 (C═N), 1498 (N═N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.05 (s, 3H, CH_3, antipyrine), 2.64 (s, 3H, CH_3, pyrazole), 2.67 (s, 2H, NH_2), 3.22 (s, 3H, CH_3–N), 7.35–7.53 (m, 5H, NH, Ar–H), 8.42 (br, 1H, NH, pyrazole), 8.52 (br, 1H, NH, hydrazone); MS (EI, 70 eV) m/z (%) = 455 ($M^+ + 1$, 21.1), 454 (M^+, 25.2), 436 (26.1), 428

(23.6), 406 (22.7), 390 (17.1), 207 (23.6), 187 (24.2), 178 (21.7), 156 (37.6), 148 (24.2), 137 (40.1), 121 (30.1), 106 (19.6), 95 (39.4), 81 (77.3), 69 (100.0), 52 (14.6). Anal. Calcd. for $C_{21}H_{18}N_{12}O$ (454.45): C, 55.50; H, 3.99; N, 36.99%. Found: C, 55.52; H, 4.07; N, 37.03%.

*4.1.3.8. 1,5-Dimethyl-4-((E)-(5-methyl-3-((E)-2-(3-methyl-5-oxo-1H-pyrazol-4(5H)-ylidene)hydrazinyl)-1H-pyrazol-4-yl)diazenyl)-2-phenyl-1H-pyrazol-3(2H)-one (**30**).* Yield (65.0%); red crystals; m.p. 278 °C; IR (KBr): ν/cm^{-1} = 3380, 3210, 3151 (3NH), 1654, 1600 (2C=O), 1498 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.12 (s, 3H, CH_3, antipyrine), 2.44 (s, 3H, CH_3, pyrazolone), 2.66 (s, 3H, CH_3, pyrazolone), 3.29 (s, 3H, CH_3–N), 7.34–7.56 (m, 5H, Ar–H, NH, pyrazole), 11.60 (br, 1H, NH, hydrazone), 12.9 (s, 1H, NH, pyrazolone); MS (EI, 70 eV) *m/z* (%) = 421 (M^+ + 1, 23.7), 420 (M^+, 24.5), 77 (27.3), 56 (100.0). Anal. Calcd. for $C_{19}H_{20}N_{10}O_2$ (420.43): C, 54.28; H, 4.79; N, 33.32%. Found: C, 54.33; H, 4.84; N, 33.37%.

*4.1.3.9. Cyanic N′-(4-((E)-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-5-methyl-1H-pyrazol-3-yl)-2-oxo-2-phenylacetohydrazonic thio-anhydride (**33**).* Yield (68.0%); dark brown crystals; m.p. 137 °C; IR (KBr): ν/cm^{-1} = 3380, 3332 (2NH), 2150 (CN), 1660 (C=O), 1488 (2N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.29 (s, 3H, CH_3, antipyrine), 2.46 (s, 3H, CH_3, pyrazole), 3.28 (s, 3H, CH_3–N), 7.38–7.56 (m, 7H, Ar–H, 2NH); MS (EI, 70 eV) *m/z* (%) = 480 (M^+ − H_2O, 36.2), 221 (34.2), 167 (3.4), 135 (34.5), 77 (100.0), 51 (32.2). Anal. Calcd. for $C_{24}H_{21}N_9O_2S$ (499.55): C, 57.70; H, 4.24; N, 25.23%. Found: C, 57.64; H, 4.22; N, 25.18%.

*4.1.4. General procedure for the synthesis of pyrazolotriazines **15**–**18**, **23**, **24**, **28** and thiadiazole **35***

A suspension of arylazo derivatives **11**–**14**, **21**, **22**, **26** and **33** (5 mmol) in acetic acid (10 mL) was refluxed for 7 h. The reaction mixture was poured into ice cold water then the formed precipitate was filtered off, dried and recrystallized from DMF/ethanol to give compounds **15**–**18**, **23**, **24**, **28** and **35**, respectively.

*4.1.4.1. 4-Amino-8-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-7-methylpyrazolo[5,1-c][1,2,4]triazine-3-carbonitrile (**15**).* Yield (65.0%); orange crystals; m.p. 301 °C; IR (KBr): ν/cm^{-1} = 3407, 3386, 3380 (NH, NH_2), 2210 (CN), 1658 (CO), 1603 (C=N), 1492 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.49 (s, 3H, CH_3, antipyrine), 2.62 (s, 3H, CH_3, pyrazole), 3.24 (s, 3H, CH_3–N), 7.34–7.58 (m, 7H, Ar–H, NH_2); MS (EI, 70 eV) *m/z* (%) = 385 (M^+ − 3H, 6.7), 297 (10.9), 161 (16.4), 132 (9.7), 93 (100.0), 77 (61.2), 56 (75.3), 55 (55.5). Anal. Calcd. for $C_{18}H_{16}N_{10}O$ (388.39): C, 55.66; H, 4.15; N, 36.06%. Found: C, 55.72; H, 4.23; N, 36.13%.

*4.1.4.2. Ethyl 4-amino-8-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl) diazenyl)-7-methylpyrazolo[5,1-c][1,2,4]triazine-3-carboxylate (**16**).* Yield (74.3%); dark brown crystals; m.p. 227–230 °C; IR (KBr): ν/cm^{-1} = 3386, 3280, 3259 (NH_2, NH), 1716, 1689 (2C=O), 1616 (N=C), 1494 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 1.36 (t, 3H, CH_3, *J* = 7.5 Hz), 2.50 (s, 3H, CH_3, antipyrine), 2.67 (s, 3H, CH_3, pyrazole), 3.37 (s, 3H, CH_3–N), 4.27 (q, 2H, O–CH_2, *J* = 7.5 Hz), 7.39–7.57 (m, 7H, Ar–H, NH_2), 8.3 (br, 1H, NH, pyrazole); MS (EI, 70 eV) *m/z* (%) = 389 (M^+ –EtOH, 16.6), 388 (25.2), 373 (24.3), 350 (23.3), 339 (19.2), 331 (19.2), 311 (25.2), 302 (18.2), 292 (23.6), 279 (22.4), 71 (53.4), 57 (100.0), 55 (59.1). Anal. Calcd. for $C_{20}H_{21}N_9O_3$ (435.44): C, 55.17; H, 4.86; N, 28.95%. Found: C, 55.24; H, 4.92; N, 28.97%.

*4.1.4.3. 4-((4-Amino-3-(1-iminoethyl)-7-methylpyrazolo[5,1-c][1,2,4]triazin-8-yl)diazenyl)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**17**).* Yield (75.0%); dark brown crystals; m.p. >300 °C; IR (KBr): ν/cm^{-1} = 3440, 3426 (NH_2, 2NH), 1633 (br, CO, C=N), 1490 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.49 (s, 3H, CH_3–CO), 2.51 (s, 3H, CH_3, antipyrine), 2.64 (s, 3H, CH_3, pyrazole), 3.35 (s, 3H, CH_3–N), 7.35–7.51 (m, Ar–H, NH_2); MS (EI, 70 eV) *m/z* (%) = 404 (M^+, 0.8), 403 (M^+ − 1, 2.4), 175 (100.0), 146 (16.3), 130 (4.9), 119 (12.8), 105 (21.9), 96 (52.1), 77 (68.9), 67 (61.9), 52 (28.1). Anal. Calcd. for $C_{19}H_{20}N_{10}O$ (404.43): C, 56.43; H, 4.98; N, 34.63%. Found: C, 56.48; H, 5.04; N, 34.67%.

*4.1.4.4. 4-((4-Amino-3-(benzo[d]thiazol-2-yl)-7-methylpyrazolo[5,1-c][1,2,4] triazin-8-yl)diazenyl)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**18**).* Yield (63.0%); yellow crystals; m.p. 252–254 °C; IR (KBr): ν/cm^{-1} = 3446, 3424 (br, NH), 1650 (CO), 1629 (C=N), 1492 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.58 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 3.35 (s, 3H, CH_3–N), 7.38–8.19 (m, 11H, NH_2, Ar–H); MS (EI, 70 eV) *m/z* (%) = 496 (M^+, 14.5), 255 (20.9), 84 (49.2), 77 (100.0), 57 (86.3). Anal. Calcd. for $C_{24}H_{20}N_{10}OS$ (496.55): C, 58.05; H, 4.06; N, 28.21%. Found: C, 58.11; H, 4.15; N, 28.28%.

*4.1.4.5. 4-((3-Acetyl-4,7-dimethylpyrazolo[5,1-c][1,2,4]triazin-8-yl) diazenyl)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**23**).* Yield (67.0%); dark brown crystals; m.p. >300 °C; IR (KBr): ν/cm^{-1} = 1670, 1637 (2CO), 1492 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.18 (s, 3H, CH_3–CO), 2.6 (s, 3H, CH_3, triazine), 2.69 (s, 3H, CH_3, antipyrine), 2.85 (s, 3H, CH_3, pyrazole), 3.38 (s, 3H, CH_3–N), 7.39–7.59 (m, 5H, Ar–H); MS (EI, 70 eV) *m/z* (%) = 406 (M^+ + 2, 4.5), 405 (M^+ + 1, 11.1), 404 (M^+, 40.8), 77 (12.0), 56 (100.0), 55 (9.2). Anal. Calcd. for $C_{20}H_{20}N_8O_2$ (404.43): C, 59.40; H, 4.98; N, 27.71%. Found: C, 59.47; H, 5.03; N, 27.76%.

*4.1.4.6. 3-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)-2-methyl-8,9-dihydrobenzo[e]pyrazolo[5,1-c][1,2,4]triazin-6(7H)-one (**24**).* Yield (66.0%); black crystals; m.p. >300 °C; IR (KBr): ν/cm^{-1} = 1646, 1637 (2C=O), 1500 (N=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 1.65–1.68 (m, 2H, CH_2, cyclohexanone), 2.45–2.72 (m, 10H, 2CH_3, 2CH_2, cyclohexanone), 3.42 (s, 3H, CH_3–N), 7.36–7.58 (m, 5H, Ar–H); MS (EI, 70 eV) *m/z* (%) = 400 (M^+ − CH_4, 42.2), 296 (48.8), 212 (43.8), 203 (47.1), 72 (100.0), 59 (17.8), 51 (16.5). Anal. Calcd. for $C_{21}H_{20}N_8O_2$ (416.44): C, 60.57; H, 4.84; N, 26.91%. Found: C, 60.62; H, 4.88; N, 26.96%.

*4.1.4.7. 6,8-Diamino-3-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl) diazenyl)-2-methylpyrazolo[5,1-c]pyrido[2,3-e][1,2,4]triazine-7-carbonitrile (**28**).* Yield (68.0%); dark brown crystals; m.p. 300 °C; IR (KBr): ν/cm^{-1} = 3294, 3151 (NH_2), 2199 (CN), 1650 (CO), 1637 (CN); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.05 (s, 3H, CH_3, antipyrine), 2.61 (s, 3H, CH_3, pyrazole), 3.23 (s, 3H, CH_3–N), 3.34 (br, 4H, 2NH_2), 7.35–7.53 (m, 5H, Ar–H); MS (EI, 70 eV) *m/z* (%) = 452 (M^+ − 2H, 28.7), 436 (34.4), 418 (32.3), 79.9 (100.0), 63.90 (99.0), 54 (49.5). Anal. Calcd. for $C_{21}H_{18}N_{12}O$ (454.45): C, 55.50; H, 3.99; N, 36.99%. Found: C, 55.53; H, 4.05; N, 37.06%.

*4.1.4.8. 4-((3-(5-Benzoyl-2-imino-1,3,4-thiadiazol-3(2H)-yl)-5-methyl-1H-pyrazol-4-yl)diazenyl)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**35**).* Yield (65.0%); orange crystals; m.p. 214–216 °C; IR (KBr): ν/cm^{-1} = 3420 (br, 2NH), 1716 (CO), 1635 (br, CO, C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.54 (s, 3H, CH_3, antipyrine), 2.67 (s, 3H, CH_3, pyrazole), 3.35 (s, 3H, CH_3–N), 7.38–7.56 (m, 10H, Ar–H), 8.61 (br, 1H, NH, pyrazole), 9.34 (s, 1H, =NH); MS (EI, 70 eV) *m/z* (%) = 500 (M^+ + 1, 6.3), 464 (15.8), 77 (22.9), 56 (100.0), 53 (13.3). Anal. Calcd. for $C_{24}H_{21}N_9O_2S$ (499.55): C, 57.70; H, 4.24; N, 25.23%. Found: C, 57.78; H, 4.27; N, 25.28%.

*4.1.5. Synthesis of 4-((3,7-dimethyl-1H-dipyrazolo[5,1-c:3′,4′-e][1,2,4]triazin-6-yl)diazenyl)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**31**)*

To a suspension of **30** (0.42 g, 1 mmol) in DMF (6 mL), phosphorous oxychloride (1 g, 6 mmol) was added, the reaction mixture

was refluxed on a water bath for 12 h. The reaction mixture was poured into sodium carbonate solution then the formed precipitate was filtered off, dried and recrystallized from DMF to give **31**. Yield (63.0%); black crystals; m.p. >300 °C; IR (KBr): $\nu/cm^{-1} = 3423$ (NH), 1625 (br, C=O, C=N), 1454 (N=N); MS (EI, 70 eV) m/z (%) = 403 ($M^+ + 1$, 0.2), 81 (100.0), 63 (79.9), 55 (10.4), 50 (3.2). Anal. Calcd. for $C_{19}H_{18}N_{10}O$ (402.41): C, 56.71; H, 4.51; N, 34.81%. Found: C, 56.74; H, 4.56; N, 34.87%.

4.2. Antitumor activity

Different concentrations of the tested compounds were prepared (ED_{100}, ED_{50} and ED_{25} μg/DMSO). The amount of DMSO was adjusted to give a final concentration of 0.1%. Ascites fluid was obtained from the peritoneal cavity of the donor animal from (National Cancer Institute, Cairo, Egypt) contain Ehrlich cell was as aseptically aspirated. The cells were grown partially floating and attach in a suspension culture (RPMI 1660 medium, Sigma Chemical Co. St. Louis, USA), supplemented with 10% fetal bovine serum (GIBCO, UK). They were maintained at 37 °C in humidified atmosphere with 5% CO_2 for 2 h. The viability of the cell used in control experiments (DMSO only without drug) exceeded 95% as determined by microscopically examination using a hemocytometer and trypan blue stain (stain only the dead cells).

4.3. Cytotoxicity and antitumor evaluation

4.3.1. Materials and methods

The reagents RPMI-1640 medium (Sigma Co., St. Louis, USA) Fetal Bovine serum (GIBCO, UK) and the cell lines HepG2, WI 38, VERO, MCF-7 were obtained from ATCC.

4.3.2. Procedure

The stock samples were diluted with RPMI-1640 medium to desired concentrations ranging from 10 to 1000 mg/mL. The final concentration of dimethylsulfoxide (DMSO) in each sample did not exceed 1% v/v. The cytotoxic activity of the compounds was tested against HepG2, WI 38, MCF-7 and VERO. The % viability of cell was examined visually. 5-Fluorouracil was used as a standard anticancer drug for comparison. Briefly, cell were batch cultured for 10 d, then seeded in 96-well plates of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Shedon.TC2323. Cornelius, OR, USA). The medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentrations of (1000, 500, 200, 100, 50, 20, 10 mg/mL). Cells were suspended in RPMI-1640 medium, 1% antibiotic–antimycotic mixture (104 μg/mL potassium penicillin, 104 mg/mL streptomycin sulfate and 25 mg/mL Amphotericin B) and 1% L-glutamine in 96-well flat bottom microplates at 37 °C under 5% CO_2. After 96 h of incubation, the medium was again aspirated, trays were inverted onto a pad of paper towels, the remaining cells rinsed carefully with medium, and fixed with 3.7% (v/v) formaldehyde in saline for at least 20 min. The fixed cells were rinsed with water, and examined. The cytotoxic activity was identified as confluent, relatively unaltered mono-layers of stained cells treated with compounds.

4.3.3. Calculation of the IC_{50} for each compound

Cytotoxicity was estimated as the concentration that caused approximately 50% loss of monolayer. The assay was used to examine the newly synthesized compounds. 5-Fluorouracil was used as a positive control. To calculate IC_{50}, you would need a series of dose–response data (*e.g.*, drug concentrations $x1$, $x2$, xn and growth inhibition $y1$, $y2$, yn). The values of y are in the range of 0–1.

4.3.4. Linear regression

The simplest estimate of IC_{50} is to plot $x - y$ and fit the data with a straight line (linear regression). IC50 value is then estimated using the fitted line, *i.e.*,

$$Y = a^*X + b; IC_{50} = (0.5 - b)/a$$

4.4. Antioxidant assay

4.4.1. Antioxidant activity screening assay; ABTS method

Antioxidant activity determinations were evaluated from the bleaching of ABTS derived radical cations. The radical cation was derived from ABTS [2,2′-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid)] was prepared by reaction of ABTS (60 μl) with MnO_2 (3 mL, 25 mg/mL) in phosphate buffer solution (10 μM, pH 7, 5 mL) After shaking the solution for a few minutes, it was centrifuged and filtered. The Absorbance (A control) of the resulting green–blue solution (ABTS radical solution) was recorded at λ_{max} 734 nm. The absorbance (A test) was measured upon the addition of (20 μl of 1 mg/mL) solution of the tested sample in spectroscopic grade MeOH/buffer (1:1 v/v) to the ABTS solution. The decrease in the absorbance is expressed as % inhibition which calculated from this equation:

$$\%\,\text{Inhibition} = [A(\text{control}) - A(\text{test})/A(\text{control})] \times 100$$

Ascorbic acid (20 μl, 2 mM) solution was used as standard antioxidant (positive control). Blank sample was run using solvent without ABTS (Table 2).

4.4.2. Bleomycin-dependent DNA damage assay [38–40]

To the reaction mixtures in a final volume of 1.0 mL, the following reagents at the final concentrations stated were added: DNA (0.2 mg/mL), bleomycin (0.05 mg/mL), $FeCl_3$ (0.025 mM), magnesium chloride (5 mM), KH_2PO_4/KOH buffer pH 7.0 (30 mM) and ascorbic acid (0.24 mM) or the test fractions diluted in MeOH to give a concentration of (0.1 mg/mL). The reaction mixtures were incubated in a water bath at 37 °C for 1 h. At the end of the incubation period, 0.1 mL of ethylenediaminetetraacetic acid (EDTA) (0.1 M) was added to stop the reaction (the iron EDTA complex is unreactive in the bleomycin assay). DNA damage was assessed by adding 1 mL 1% (w/v) thiobarbituric acid (TBA) and 1 mL of 25% (v/v) hydrochloric acid (HCl) followed by heating in a water-bath maintained at 80 °C for 15 min. The chromogen formed was extracted into 1-butanol, and the absorbance was measured at 532 nm.

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