Convenient Synthesis, Characterization, Cytotoxicity and Toxicity of Pyrazole Derivatives

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Abstract:

3-Methyl-1*H*-pyrazol-5(*4H)*-one (**1**) was used as a template to develop new anticancer compounds and investigate their SAR. The ring modification of compound **1** occurred through its reaction with aromatic aldehydes and different reagents to afford the corresponding 6-oxopyrano[2,3-*c*]pyrazoles**4a-c** and their amino analogues 6-aminopyrano[2,3-*c*]pyrazoles**6a-c, 8**; the pyrazolopyrano[2,3-*b*]pyridines **10a-c** and the chromenopyrano[2,3-*c*]pyrzolones**13, 14**. The reaction of compound **1** with thiourea and appropriate aromatic aldehydes afforded the pyrazolo[3,4-*d*]pyrimidine derivatives **17a-c**. On the other hand, the pyrazolo[3,4-*d*]thiazole derivatives **22a-d** were obtained via the reaction of **1** with sulpher and aryl isothiocyanates in presence of triethylamine. The reaction of compound **1** with phenylisothiocyanate followed by treatment with the α-halocarbonyl compounds **24a-c** afforded the thiazole derivatives **25a-c**. The synthesized products were evaluated for their cytotoxicity against cancer and normal cell lines. Most compounds showed significant anticancer activity without affecting the normal fibroblast cells. The toxicity of the mostpontent cytotoxic compounds was measured using Brine-Shrimp Lethality Assay.

Keywords: pyrazole; pyrano[2,3-*c*]pyrazole; pyrazolo[3,4-*d*]pyrimidine; pyrazolo[3,4-*d*]thiazole; cytotoxicity

1. Introduction

Cancer is a major public health problem in the world. Chemotherapy is still one of the primary modalities for the treatment of cancer. However, the use of this method is limited mainly due to the small number of the available chemotherapeutic agents to choose among them and also because the use of these agents is often accompanied by undesirable side effects. This clearly underlies the urgent need for developing novel chemotherapeutic agents with more potent antitumor activities and reduced side effects. Many pyrazole derivatives have attracted considerable attention in the recent years for their diverse biological activities.[1–6] They are also acknowledged for their anticancer activities.[7-9] Celecoxib (1), Sulfaphenazole (2), CDPPB (3), Linazolac (4), Mepiprazole (5), and Rimonabant (6) are some of the pyrazole based drugs available today in the market (Fig. 1).[10]

Moreover, the chemistry of fused pyrazole derivatives has drawn great attention due to their pharmacological importance.[11,12] It has been found that pyranopyrazoles are an important class of biologically active heterocycles. They are reported to possess a multiplicity of pharmacological properties including anticancer,[13] antimicrobial,[14] anti-inflammatory,[15] insecticidal and molluscicidal ctivities.[16,17] They are also potential inhibitors of human Chk1 kinase.[18] On the otherhand, pyrazolopyrimidines which are the fused heterocyclic ring systems that structurally resemble purines, prompted biological investigations to assess their potential therapeutic significance. They are known to play a crucial role in numerous disease conditions. The collective results of biochemical and biophysical properties foregrounded their medicinal significance in central nervous system, cardiovascular system, cancer and inflammation.[19-21] In addition, several 1,3-thiazole scaffolds have been reported as potent anticancer agents.[22-24] The synthesis of some new pyrazole-based 1,3-thiazoles as anticancer agents was reported.[25] Most recently, excellent anticancer effectiveness of pyrazolylthiazole derivatives was also reported, via EGFR TK inhibition that plays an important role in cell growth regulation.[26] However reviewing the literature and to our knowledge, the discovery of the potential anticancer activity of pyrazolothiazoles is still essentially in the development stage. In view of the aforementioned facts, our efforts were directed towards the uses of 3-methyl-1*H*-pyrazol-5(4*H*)-one to prepare heterocyclic and fused derivatives together with evaluation of their activity towards cancer and normal cell lines.

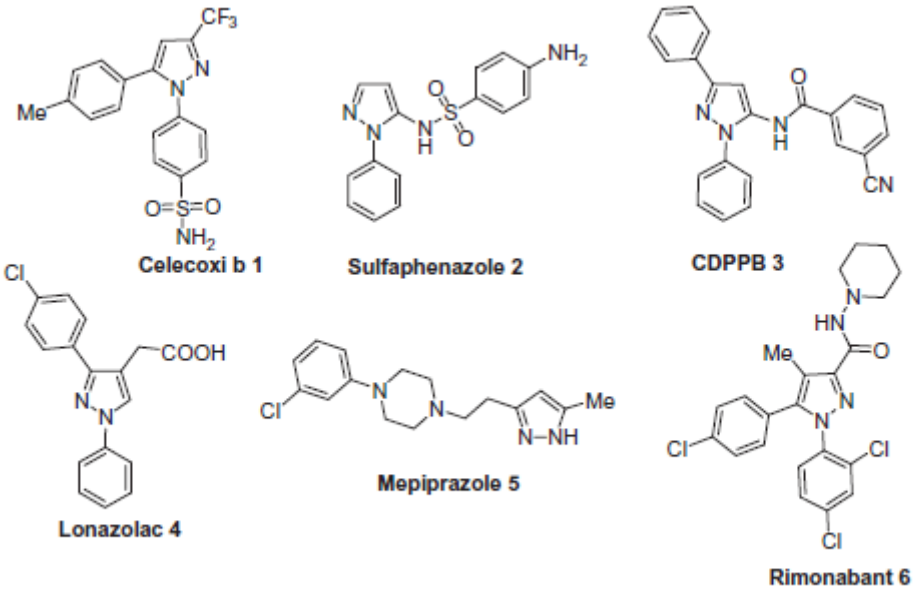


Figure 1. Biologically active pyrazole derivatives.

2. Results and Discussion

2.1. Chemistry

The present investigation emphasized mainly on the synthesis of molecules derived from pyrazole-5-one and evaluation of their cytotoxicity against cancer and normal cell lines. The synthetic strategies adopted for the synthesis of the intermediate and target compounds are depicted in Schemes 1-4. One pot multicomponent reactions (MCR) were utilized to prepare the target compounds. The reaction of the 3-methyl-1*H*-pyrazol-5(4*H*)-one (**1**)with each of benzaldehyde (**2a**), 4-methoxybenzaldehyde (**2b**) or 4-chlorobenzaldehyde (**2c**) and ethyl cyanoacetate (**3**) afforded the 6-oxopyranopyrazole derivatives **4a-c**. Structures of the latter products were confirmed on the basis of their respective analytical and spectral data. Thus, ^{1}HNMR spectrum of compound **4a** revealed the presence of a singlet at δ 2.49 ppm indicating the presence of the CH_3 group, a multiplet at δ 7.59-8.41 ppm equivalent to the C_6H_5 group and a singlet at δ 10.40 ppm corresponding to the NH group. Meanwhile, the reaction of compound **1** with either of **2a**, **2b** or **2c** and malononitrile (**5**) in ethanol containing triethylamine gave the 6-amino-3-methyl-4-aryl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile derivatives **6a-c**, respectively. The analytical and spectral data of **6a-c** were in consistence with their respective

structures. The latter compounds were previously reported to be prepared via a one pot, four component reaction between aldehydes, hydrazine hydrate, malononitrile and ethyl acetoacetate in presence of different catalysts.[27] On the other hand, the reaction of compound **1** with pyridine-3-aldehyde (**7**) and malononitrile afforded the 6-amino-3-methyl-4-(pyridin-3-yl)-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile **8**. The structure of the latter product was based on their respective analytical and spectral data. Thus, the ^{1}H NMR spectrum showed the presence of a singlet at δ 1.79 ppm indicating the CH_3 group, a singlet at δ 4.69 ppm equivalent to the pyran H-4, a singlet at δ 6.95 ppm for the NH_2 group and a multiplet at δ 7.32-8.46 ppm corresponding to the pyridine protons.

Moreover, the reaction of **1** with the aromatic aldehydes **2a-c** and 2-aminoprop-1-ene-1,1,3-tricarbonitrile (**9**) in ethanol containing a catalytic amount of triethylamine afforded the pyrazolopyrano[2,3-*b*]pyridine-6-carbonitrile derivatives **10a-c**. ^{1}H NMR of compound **10a** (as an example) showed the presence of a singlet at δ 2.49 ppm corresponding to the CH_3 group, a singlet at δ 4.58 ppm for the pyran H-4, two singlets at δ 7.10 and 8.02 ppm indicating the presence of the two NH_2 group. Moreover, ^{13}C NMR showed signals at δ 36.9 indicating the pyran C-4 and signals at δ114.1, 127.1, 128.3, 129.1, 137.3, 144.9, 146.8, 148.4, 150.6, 154.3, 154 equivalent to the phenyl, pyrazole, pyran and pyridine C's. On the other hand, the reaction of the compound **6b** with phenylisothiocyanate (**11**) in 1,4-dioxane afforded the corresponding thiourea derivative **12,** the structure of which was based on analytical and spectral data.

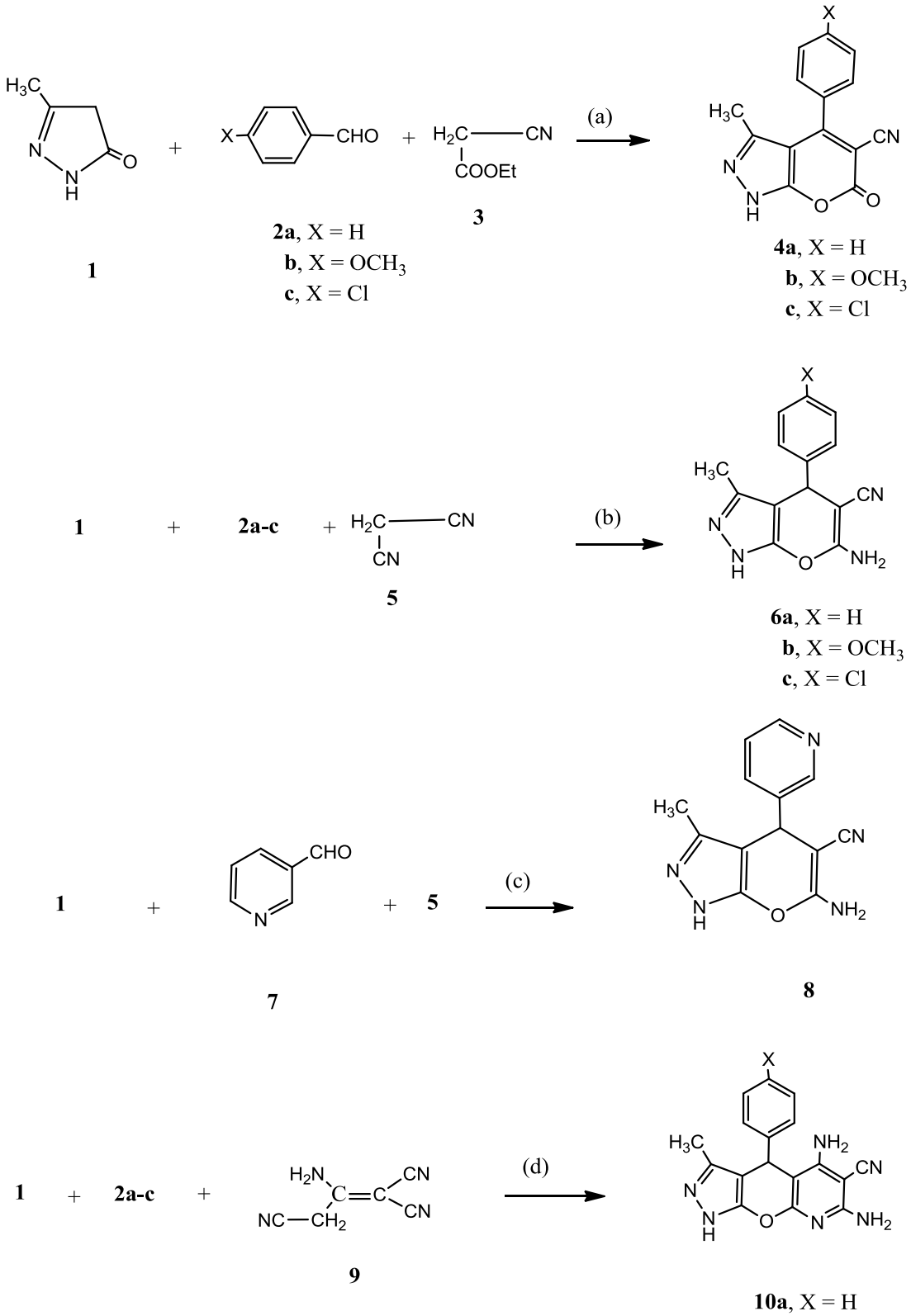
The one-pot reaction of compound **1** with salicylaldehyde and malononitrilegave the annulated 5-amino-1-methyl-3*H*-chromeno[4',3':4,5]-pyrano[2,3-*c*]pyrazol-6(11*bH*)-one (**13**). The analytical and spectral data of the latter product was the basis of their structural elucidation. Thus, the ^{1}H NMR spectrum of **13** showed, beside the expected signals, the presence of a singlet at δ 4.14 ppm indicating the NH_2 group, a multiplet at δ 7.29-7.57 ppm corresponding to the C_6H_4 group and a singlet at δ 11.01 ppm (D_2O exchangeable) for the NH group. Moreover, the ^{13}C NMR spectrum showed δ 162.0, 162.5, 163.0 indicating the C=N and C=O groups. Similarly, the reaction of compound **1** with salicylaldehyde and ethyl cyanoacetate furnished the 1-methyl-3*H*-chromeno[4',3':4,5]pyrano[2,3-*c*]pyrazole-5,6-dione (**14)**.

The multi-component reaction [MCR] of compound **1** with thiourea and aromatic aldehydes was investigated. Thus, the one-pot reaction of the pyrazole **1** with thiourea (**15**) and either benzaldehyde (**2a**), 4-methoxybenzaldehyde (**2b**) or 4-bromobenzaldehyde (**16**) in the presence of triethylamine gave the pyrazolo[3,4-*d*]pyrimidine derivatives **17a-c**. The structure of the synthesized compounds was confirmed via the analytical and spectral data (see experimental section).

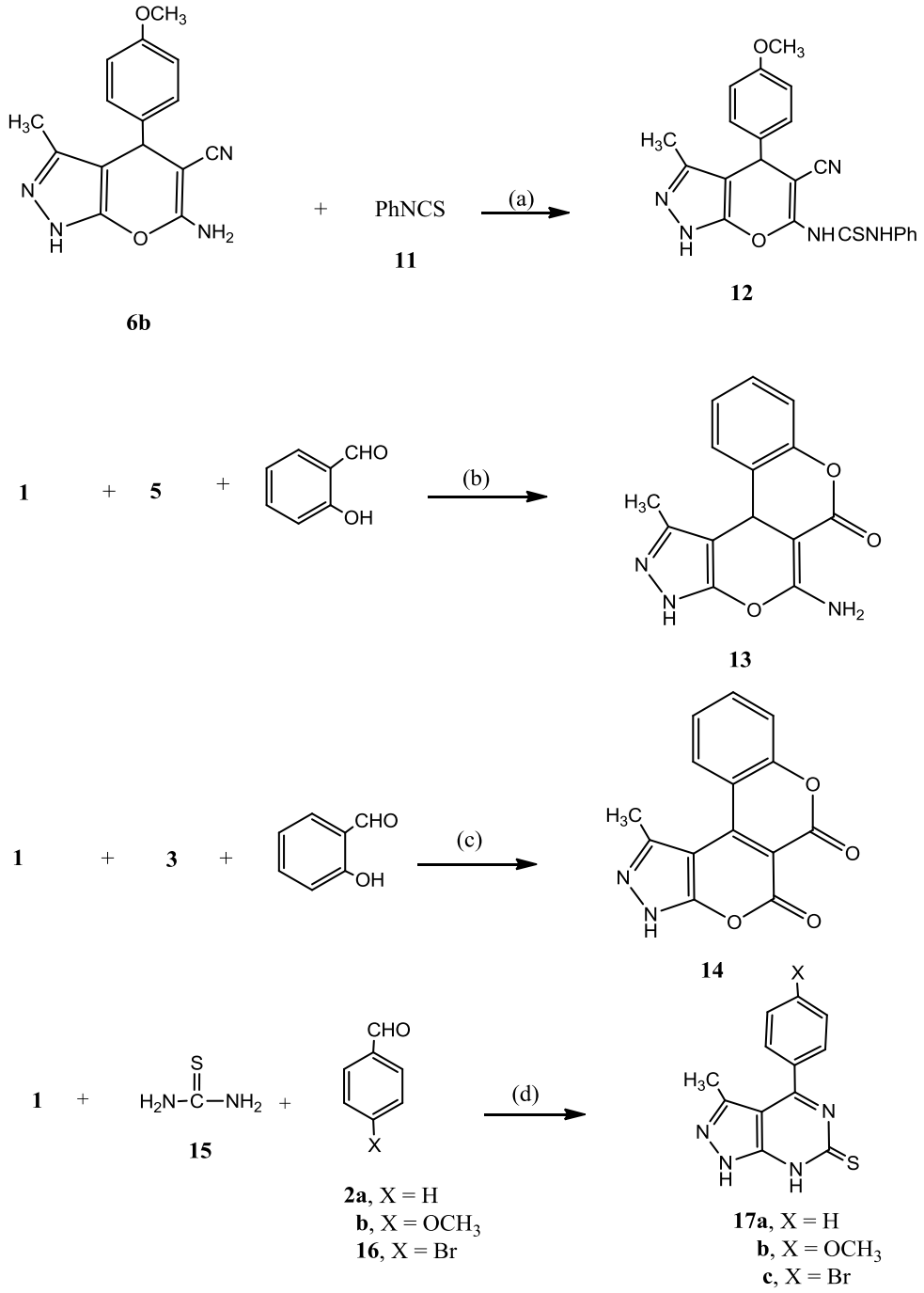
Reaction of compound **1** with triethylorthoformate (**18**) in an oil bath at 120 ^{o}C afforded the 4-(ethoxymethylene)-3-methyl-1*H*-pyrazol-5(4*H*)-one (**19**). The structure of compound **19** was established on the basis of analytical and spectral data. Thus, the ^{1}H NMR spectrum showed a triplet and quartet at δ 1.29 and 4.15 ppm corresponding to the ethyl group and a singlet at δ 7.38 ppm indicating CH=C group. The reaction of compound **1** with malononitrile and triethylorthoformate in ethanol gave compound **20**. The presence of the two CN groups was indicated by the presence of two absorption bands at IR spectrum at ν 2204, 2179 cm^{-1}. ^{1}H NMR spectrum showed a singlet at δ 8.66 ppm corresponding to the CH=C group. Further confirmation of the structure of compound **20** was obtained through its synthesis via another reaction route. Thus, the reaction of malononitrile (**5**) with compound **19** gave the same product **20** (m.p. and mixed m.p. and finger print IR). Moreover, the reaction of compound **1** with elemental sulfur and either phenylisothiocyanate (**11**), 4-methoxyphenylisothiocyanate (**21a**), 4-chlorophenylisothiocyanate (**21b**), or 4-bromophenylisothiocyanate (**21c**) in 1,4-dioxane containing triethylamine gave the pyrazolo[3,4-*d*]thiazole derivatives **22a-d**. The structures of the latter products were based on the analytical and spectral data. Thus, the ^{1}H NMR spectrum of **22a** (as an example) showed the presence of a singlet at δ 2.49 ppm corresponding to CH_3 group, a mutiplet at d 7.09-7.50 ppm corresponding to the phenyl protons and a singlet at δ 9.75 equivalent to the NH group. Moreover, the ^{13}C NMR spectrum showed the presence of the CH_3 group at δ 12.27, the phenyl and pyrazole C's at δ 124.5, 128.5,128.9, 129.4, 130.4, 137.8, 139 and the C=S group at δ 180.1.

The methylene group present in the pyrazole **1** showed high reactivity towards thiazole formation via its reaction with phenylisothiocyanate in basic DMF solution followed by heterocyclization with α-halocarbonyl compounds.[28,29] Thus, compound

1 was reacted with phenylisothiocyanate in DMF/KOH solution to give the intermediate potassium sulphide salt **23**. The reaction of the latter intermediate with either 2-bromo-1-phenylethanone (**24a**), 2-bromo-1-(4-chlorophenyl)ethanone (**24b**) or ethyl chloroacetate (**24c**) gave the thiazole derivatives **25a-c**. The structures of the latter products were established on the basis of their respective analytical and spectral data.



Scheme 1. Synthesis of pyrazole derivatives **4a-c**, **6a-c, 8** and **10a-c;** reagents and conditions: (a) EtOH/Et_3N, heat 1 h (b) EtOH/Et_3N, heat 1 h (c) EtOH/Et_3N, heat 2h (d) EtOH/Et_3N, heat 1 h



Scheme 2. Synthesis of pyrazole derivatives **12-14** and **17a-c;** reagents and conditions: (a) 1,4 dioxane/Et_3N, heat 2 h (b) EtOH/Et_3N, heat 2 h (c) EtOH/Et_3N, heat 2 h (d) EtOH/Et_3N, heat 1 h

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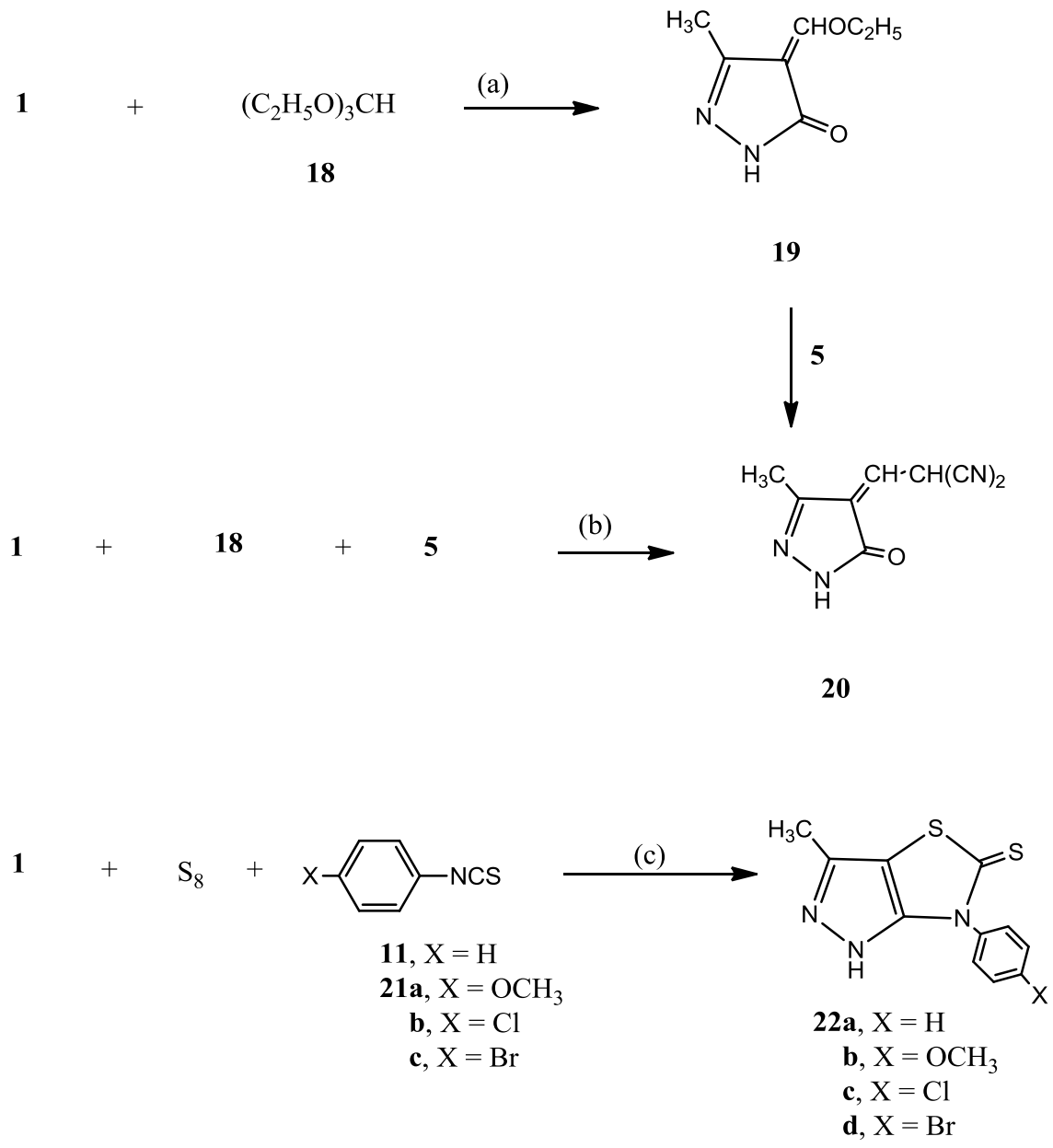
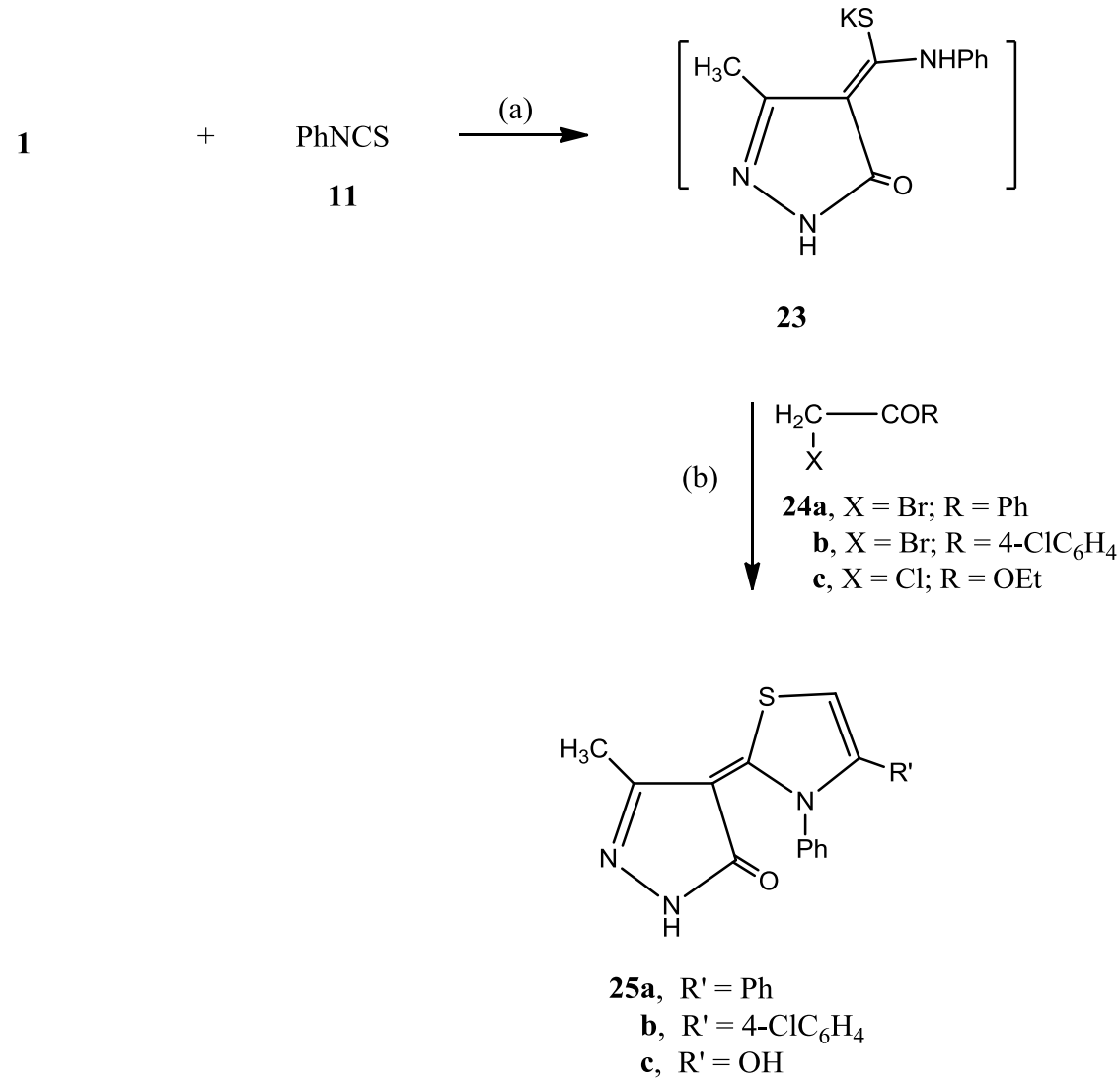


Scheme 3. Synthesis of pyrazole derivatives **19**, **20**, **22a-d**.; reagents and conditions: (a) fusion 120 C, 30 min (b) EtOH/Et_3N, heat 2 h (c) 1,4-dioxane/Et_3N, heat 3 h



Scheme 4. Synthesis of pyrazole derivatives **25a-c,** reagents and conditions: (a) DMF/KOH, r.t. (b) r.t. overnight

2.2. *In vitro* cytotoxicity

2.2.1. Effect on the Growth of Human Cancer Cell Lines

The heterocyclic compounds, prepared in this study, were evaluated according to standard protocols for their *in vitro* cytotoxicity against six human cancer cell lines including cells derived from human gastric cancer (NUGC), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), nasopharyngeal carcinoma (HONE1), human breast cancer (MCF) and normal fibroblast cells (WI38). For comparison reasons, CHS 828 was used as standard anticancer drug. All of IC_{50} values in (nM) are listed in Table 1 and the results are presented graphically in Figures: 1, 2, 3.

Many of the synthesized heterocyclic compounds were observed with significant cytotoxicity against most of the cancer cell lines tested (IC_{50}<1000 nM). Normal fibroblasts cells (WI38) were affected to a much lesser extent (IC_{50}>10,000 nM). Among the tested compounds the 3-methyl-6-phenyl-1*H*-pyrazolo[3,4-*d*]thiazole-5(6*H*)-thione**(22a)** was found to show the highest cytotoxic effect against the 6 cancer cell lines in the range of IC_{50} 33-442 nM. Broad spectrum antitumor activity was exhibited by compounds **4c**, **6b**, **10b**, **12**, **17b**, **19**, **22a**, **22b** and **22d**. Several compounds showed potent cytotoxic effect with IC_{50} < 100 nM for example compounds: **8**, **10c**, **12**, **22a, 22d** against NUGC; **10b**, **10c**, **17b**, **19**, **20**, **22a, 22b, 22d** against DLD1; **6a**, **17b**, **19**, **22a, 22d** against HA22T, **17b** against HEPG2 and **22a** against MCF.

2.2.2. Structure activity relationship

In the present study, a series of heterocyclic derivatives incorporating a pyrazole moiety were synthesized and evaluated for their cytotoxicty aiming at investigating their SAR. Thus 6-oxopyranopyrazole derivatives **4a-c** and their amino analogs **6a-c** and **8** were prepared. Refering to the IC_{50} values listed in table 1, compound **4a** bearing a phenyl substituent exhibited significant broad spectrum cytotoxic activity in the range of (IC_{50} 120-527 nM). Meanwhile, **4b** bearing a 4-$OCH_3C_6H_4$ group showed selective activity against liver cancer HEPG2 (IC_{50} 428 nM) and breast cancer MCF (IC_{50} 580 nM). The 4-ClC_6H_4 substituted derivative **4c** demonstrated better activity compared to **4a** and **4b** especially against gastric cancer NUGC (IC_{50} 60 nM). Among the 6-amino-4-substitutedpyranopyrazole derivatives **6a-c** and **8,** derivative **6a** carrying a 4-C_6H_5 group was found to have selective activity against the human liver cancer cell line HEPG2 (IC_{50} 399 nM) and colon cancer cell line DLDI (IC_{50} 890 nM). However compound **6b** bearing 4-$OCH_3C_6H_4$ group was completely deviod of cytotoxic acvtivity. On the other hand, compound **6c** bearing the 4-ClC_6H_4 moiety showed high activity against all cancer cell lines except breast cell line MCF in the range of (IC_{50} 120-359 nM). The presence of pyridine ring in compound **8** is most probably responsible for its high potency against human liver cancer cell line HA22T (IC_{50} 58 nM) and nasopharyngeal cancer cell line HONE1 (IC_{50} 180 nM). The previous results suggests that the replacement of the 6-amino group in compounds **6a-c** by a 6-oxo group in compounds **4a-c** in the latter pyranopyrazole derivatives led to compounds with enhanced cytotoxic effect which

might be attributed the presence of the electronegative oxygen moiety. Meanwhile, replacement of the 2-amino group of compound **6b** by a phenylthiourea moiety afforded compound **12**which demonstrated a dramatic increase in the cytotoxic activity with the highest activity exhibited against NUGC (IC_{50} 36 nM).

The investigation of the cytotoxicity of the pyrazolo[4',3':5,6]pyrano[2,3-*b*]pyridine derivatives **10a-c** revealed that compound **10a** bearing a phenyl group exhibited selective activity against MCF (IC_{50} 112 nM). On the other hand, compound **10b** bearing the 4-$OCH_3C_6H_4$ group was found to be active against most cancer cell lines with the highest activity against NUGC (IC_{50} 122 nM) and DLDI (IC_{50} 90nM). The 4-ClC_6H_4 substituted derivative **10c** showed high cytotoxic activity against four cancer cell lines with potent activity against NUGC (IC_{50} 40 nM) and DLDI (IC_{50} 60 nM). Meanwhile, the tetracyclic chromenopyranopyrazole derivatives **13** and **14** were found to be almost deviod of cytotoxic activity which might be attributed to the existence of the annelated ring system. Compound **14** showed only moderate selective activity against HEPG2 (IC_{50} 410 nM).

Considering the pyrazolo[3,4-*d*]pyrimidinederivatives**17a-c,** compound **17a** bearing the un-substituted phenyl moiety was found to lack cytotoxic activity. However, replacement of the phenyl group by the 4-$OCH_3C_6H_4$ moiety in compound **17b** was accompanied by a dramatic enhancement of the activity appearing through its high activity against the six cancer cell lines with significant cytotoxicity against human liver cancer cell line HA22T (IC_{50} 42 nM), HEPG2 (IC_{50} 59 nM) and DLDI (IC_{50} 66 nM). Meanwhile, compound **17c** bearing a p-BrC_6H_4 moiety showed only selective activity against breast cancer cell line MCF (IC_{50} 380 nM). On the other hand, the 4-(ethoxymethylene)-3-methyl-1*H*-pyrazol-5(4*H*)-one derivative **19** exhibited more potent cytotoxic activity than compound **20**. Such activity was demonstrated in the high cytotoxicity against 6 human cancer cell lines with highest activity against HA22T (IC_{50} 34 nM) and DLDI (IC_{50} 40 nM) which may be attributed to the presence of the ethoxymethylene moiety. Compound **20** showed selective cytotoxic effect against DLDI, NUGC and HEPG2 in the range of (IC_{50} 60-365 nM). Furthermore, the pyrazolothiazole derivatives **22a**, **22c** and **22d** exhibited potent to moderate broad spectrum activity. The results shown in table 1 reveals that 3-methyl-6-phenyl-*1H*-pyrazolo[3,4-*d*]thiazole-5(6*H*)-thione (**22a**) showed the maximum cytotoxicity among the tested compounds towards the cancer cell lines. Compound **22b** bearing a p-$OCH_3C_6H_4$showed potent cytotoxic activity against DLDI (IC_{50} 38

nM). On the other hand, the p-BrC_6H_4 substituted derivative **22d** showed almost three fold more activity than its p-ClC_6H_4 analogue **22c** against NUGC, DLD1and HA22T.

Considering the thiazole derivatives **25a-c**, it is obvious that among the three compounds, the 4-(4-hydroxy-3-phenylthiazol-2(3*H*)-ylidene)-3-methyl-1*H*-pyrazol-5(4*H*)-one (**25c**) demonstrated better cytotoxic activity compared to its analogues. Compounds **25a-c** showed potent to moderate activity against breast cancer MCF in the range of 64-260 nM. Most of the potent cytotoxic compounds affected the normal fibroblast cells W138 to a much lesser extent (IC_{50}>10,000nM).

In summary it is of great value to conclude from Table 1 that compounds **4a**, **4c**, **6c**, **10b**, **10c**, **12**, **17b**, **19**, **20**, **22a**, **22b**, **22c**, **22d** and **25c** showed the highest cytotoxicity among the tested compounds. Moreover, the thiazole derivative **22a** showed the maximum cytotoxicity among all compounds. Figures 2 represent the cytotoxicity of compounds **4a-c**, **6a-c**, **8**, **10a-c** and figure 3 for compounds **22a-d, 25a-c.**

Table1. Cytotoxicity of the synthesized compounds against a variety of cancer cell lines [a] [IC_{50} [b] (nM)].

Compd	Cytotoxocity (IC_{50} in nM)						
	NUGC	DLDI	HA22T	HEPG2	HONE1	MCF	WI38
4a	343	440	120	415	527	231	Na
4b	1280	2237	2337	428	1168	580	Na
4c	60	220	na	227	2354	228	Na
6a	1084	890	3068	399	2280	3365	Na
6b	2420	2445	3017	2320	1820	3444	2234
6c	210	120	283	359	206	2655	Na
8	1101	1180	58	2766	180	na	Na
10a	3124	2670	1165	4321	2166	112	Na
10b	122	90	212	440	1877	436	Na
10c	40	60	152	320	2280	1663	690
12	36	326	122	421	682	1293	1288
13	3255	2674	1374	2693	2227	1438	25
14	1235	3160	2168	410	2146	1263	Na

17a	2240	2388	1336	1120	1268	3844	320
17b	140	66	42	59	822	625	Na
17c	2230	3199	3163	2791	2329	380	Na
19	120	40	34	374	244	120	Na
20	180	60	3265	365	4423	2533	Na
22a	33	48	29	320	442	66	Na
22b	350	38	1169	2349	2210	169	1180
22c	112	204	282	212	192	2230	2066
22d	38	65	88	235	370	1160	Na
25a	3210	1264	1129	2231	388	64	1582
25b	2188	3285	1723	2735	1078	219	428
25c	66	1250	688	138	1109	260	360
CHS 828	25	2315	2067	1245	15	18	Na

[a]NUGC, gastric cancer; DLDI, colon cancer; HA22Tand HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer; WI38, normal fibroblast cells.

[b]The sample concentration that produces a 50% reduction in cell growth.

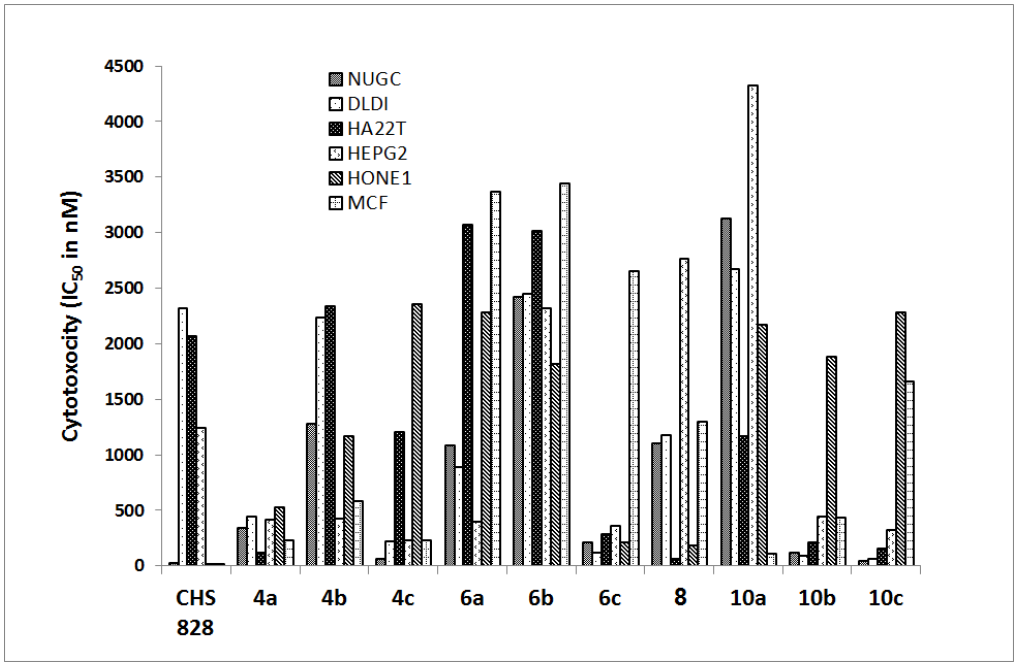


Figure 2. Cytotoxicity of compounds **4a-c**, **6a-c**, **8**, **10a-c** and CHS 828 against NUGC, gastric cancer; DLDI, colon cancer; HA22Tand HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer.

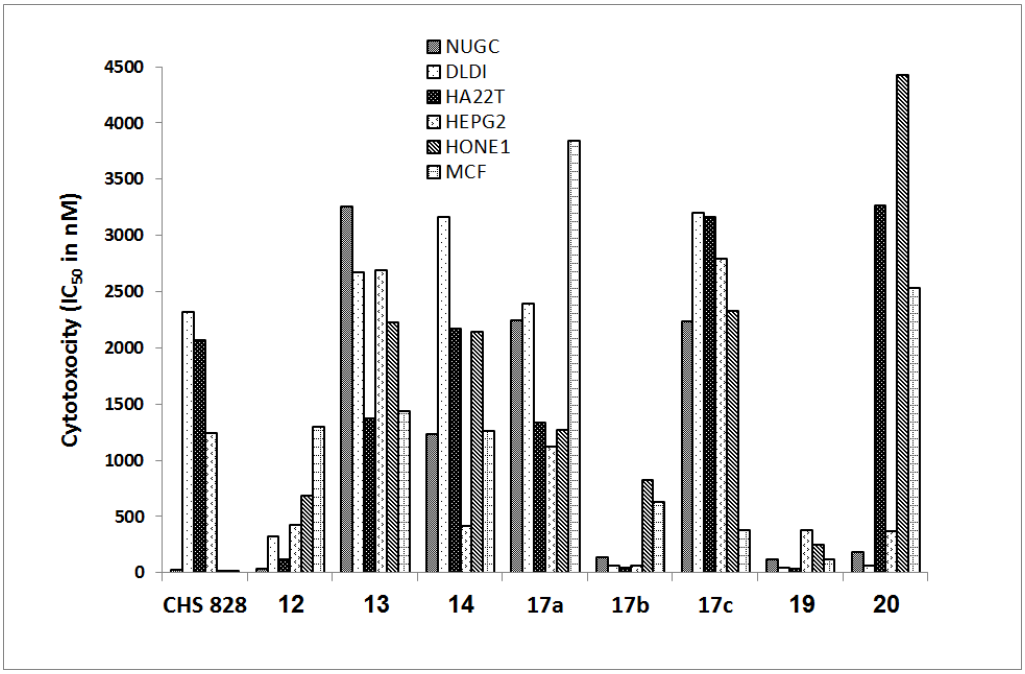


Figure 3. Cytotoxicity of compounds **12**, **13**, **14, 17a-c, 19, 20** and CHS 828 against NUGC, gastric cancer; DLDI, colon cancer; HA22T and HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer.

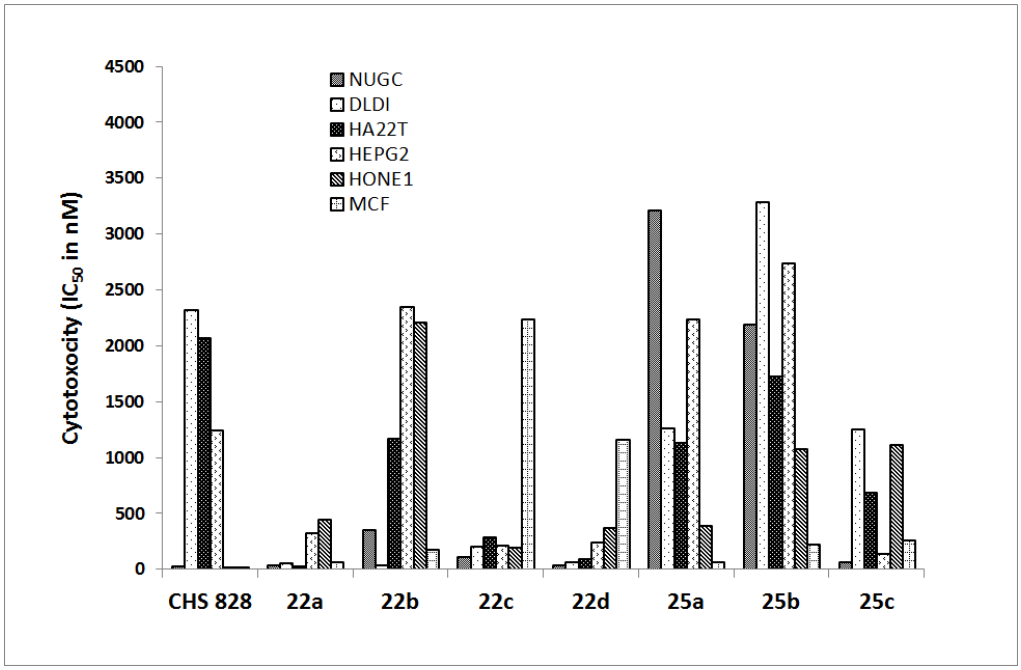


Figure 4. Cytotoxicity ofcompounds **22a-d, 25a-c** and CHS 828 against NUGC, gastriccancer; DLDI, colon cancer; HA22T and HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer

2.3 Toxicity testing

Bioactive compounds are often toxic to shrimp larvae.Thus, in order to monitor these chemicals' *in vivo* lethality toshrimp larvae (*Artemiasalina*), Brine-Shrimp Lethality Assay (Choudhary*et al.*, 2001)was used.[30] Results were analyzed with LC_{50} programto determine LC_{50} values and 95% confidence intervals.[31]Results are given in Table 2 for the compounds which exhibited optimal cytotoxic effect against cancer cell lines which are the fourteen compounds**4a**, **4c**, **6c**, **10b**, **10c**, **12**, **17b**, **19**, **20**, **22a**, **22b**, **22c**, **22d** and **25c**. The shrimp lethality assay is considered as a useful toolfor preliminary assessment of toxicity, and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticides, and cytotoxicitytesting of dental materials, natural and syntheticorganic compounds. It has also been shown that, *A. salina*toxicity test results have a correlation with rodent and human acute oral toxicity data. Generally, a good correlationwas obtained between *A. salina*toxicity test and therodent data. Likewise, the predictive screening potential ofthe aquatic invertebrate tests for acute oral toxicity in man, including *A. salina*toxicity test, was slightly better than therat test for test compounds.[32]

In order to prevent the toxicity results from possible false effects originated from solubility of compounds and DMSO'spossible toxicity effect, compounds were

prepared bydissolving in DMSO in the suggested DMSO volume ranges. It is clear from Table 2 that compounds **4a**, **6c, 17b**, **22a** and **22b** showed non toxicity against the tested organisms. It is of great value to mention that compound **22a** which is of optimum cytotoxicity is one on the non toxic compounds.

Table 2. Toxicity of the most optimal cytotoxic compounds against shrimp larvae

Compound No.	Cons. (μg/ml)	Mortality[a]	Toxicity	LC_{50}	Upper 95% lim.	Lower 95% lim
4a	10 100 1000	0 0 4	Non toxic	890.38	-	-
4c	10 100 1000	0 4 8	Harmful	14.18	560.12	160.30
6c	10 100 1000	0 0 8	Non toxic	451.19	-	-
10b	10 100 1000	5 8 10	Very toxic	112.65	469.28	230.41
10c	10 100 1000	2 4 10	toxic	100.00	104.2	157.62
12	10 100 1000	0 3 8	Harmful	14.38	220.52	140.91
17b	10 100	0 0	Non-toxic	945,21	-	-

	1000	4				
19	10 100 1000	0 6 10	toxic	80.00	290.23	70.22
20	10 100 1000	2 8 10	Very toxic	251.19	650.30	159.17
22a	10 100 1000	0 0 8	Non-toxic	890.41	-	-
22b	10 100 1000	0 2 8	Harmful	18.72	630.21	440.01
22d	10 100 1000	0 0 8	Non-toxic	1000.0	-	-
25c	10 100 1000	0 2 10	Harmful	16.38	620.22	168.34

[a]Ten organisms (*A. salina*) tested for each concentration.

3. Experimental

3.1. Chemistry

All melting points were determined on a Stuart apparatus and the values given are uncorrected. IR spectra (KBr, cm^{-1}) were determined on a Shimadzu IR 435 spectrophotometer (Faculty of Pharmacy, Cairo University, Egypt). ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Gemini 300 MHz (Microanalysisl Center, Cairo University, Egypt) and Bruker Ascend 400 MHz spectrophotometers (Microanalytical Unit, Faculty of Pharmacy, Cairo University, Egypt) using TMS as internal standard. Chemical shift values are recorded in ppm on δ scale. Mass spectra were recorded on a Hewlett Packard 5988 spectrometer (Microanalysis Center, Cairo University, Egypt). Elemental analyses were carried out at the Microanalysis Center,

Cairo University, Egypt; found values were within ±0.35% of the theoretical ones. Progress of the reactions was monitored using thin layer chromatography (TLC) sheets precoated with UV fluorescent silica gel Merck 60F 254 and were visualized using UV lamp.

3.1.1. General procedure for synthesis of compounds 4a-c and 6a-c

To a solution of compound **1** (0.98 g, 0.01 mol) and the appropriate aldehyde (0.01 mol) in ethanol (30 mL) containing triethylamine (1.0 mL) either malononitrile (0.66 g, 0.01 mol) or ethyl cyanoacetate (1.13 g, 0.01 mol) was added. The reaction mixture, in each case, was heated under reflux for 1 h, left to cool and the formed solid product, in each case, was collected by filtration and crystallized from ethanol.

3-Methyl-6-oxo-4-phenyl-1,6-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (4a). Yield: 80%; m.p.: 68-70 °C; IR (KBr, cm^{-1}) ν: 3439 (NH), 3032 (CH aromatic), 2981, 2953 (CH aliphatic), 2223 (CN), 1728 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.59–8.41 (m, 5H, Ar-H), 10.40 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 251 (M^+, 55). *Anal. calcd. for* $C_{14}H_9N_3O_2$: C, 66.93; H, 3.61; N, 16.73. Found: C, 66.75; H, 3.36; N, 16.95.

4-(4-Methoxyphenyl)-3-methyl-6-oxo-1,6-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4b**). Yield: 85%; m.p.: 75-77 °C; IR (KBr, cm^{-1}) ν: 3385 (NH), 3050 (CH aromatic), 2954, 2935 (CH aliphatic), 2216 (CN), 1722 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 6.88-8.32 (m, 4H, Ar-H), 10.42 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 281 (M^+,74). *Anal. calcd. for* $C_{15}H_{11}N_3O_3$: C, 64.05; H, 3.94; N, 14.94. Found: C, 63.90; H, 3.88; N, 14.82.

4-(4-Chlorophenyl)-3-methyl-6-oxo-1,6-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4c**). Yield: 78%; m.p.: 110-112 °C; IR (KBr, cm^{-1}) ν: 3373 (NH), 3032 (CH aromatic), 2960 (CH aliphatic), 2223 (CN), 1728 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.66–8.42 (m, 4H, Ar-H), 10.38 (s, 1H, NH, D_2O exchangeable) ppm;^{13}C NMR (DMSO-d_6, 400 MHz): 14.4, 103.4, 115.8, 128.9, 129.2,130.3, 131.6, 132.7, 138.5, 154.2, 162.1, 162.6; MS (*m/z*, %): 285 (M^+, 66 %). *Anal. calcd. for* $C_{14}H_8ClN_3O_2$: C, 58.86; H, 2.82; N, 14.71.Found: C, 58.90; H, 2.88; N, 14.45.

6-Amino-3-methyl-4-phenyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (6a). Yield: 85%; m.p.: 232-234 °C; IR (KBr, cm^{-1}) ν: 3406, 3157 (NH_2, NH), 3024 (CH aromatic), 2899, 2991 (CH aliphatic), 2017 (CN), 1635 (C=C);^{1}H NMR (DMSO-d_6) δ: 1.78 (s, 3H, CH_3), 4.58 (s, 1H, pyran H-4), 6.83 (s, 2H, NH_2, D_2O exchangeable), 7.15–7.34 (m, 5H, Ar-H), 12.06 (s, 1H, NH, D_2O exchangeable) ppm; MS (*m/z*, %): 252 (M^+, 12 %). *Anal. calcd. for* $C_{14}H_{12}N_4O$: C, 66.65; H, 4.79; N, 22.21. Found: C, 66.38; H, 4.91; N, 21.95

6-Amino-4-(4-methoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyazole-5-carbonitrile (6b). Yield: 89%; m.p.: 210-212°C; IR (KBr, cm^{-1}) ν: 3483, 3255 (NH_2, NH), 3107 (CH aromatic), 2960, 2912 (CH aliphatic), 2191 (CN); ^{1}H NMR (DMSO-d_6) δ: 1.78 (s, 3H, CH_3), 3.72 (s, 3H, OCH_3), 4.53 (s, 1H, pyran H-4), 6.85 (s, 2H, NH_2, D_2O exchangeable), 6.87-7.09 (m, 4H, Ar-H), 12.04 (s, 1H, NH, D_2O exchangeable) ppm; MS (*m/z*, %): 282 (M^+, 20 %). *Anal. calcd. for* $C_{15}H_{14}N_4O_2$:C, 63.82; H, 5.00; N, 19.85. Found: C, 63.50, H, 4.79, N 19.67.

6-Amino-4-(4-chlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (6c). Yield: 82%; m.p.: 234-236 °C; IR (KBr, cm^{-1}) ν: 3479, 3234 (NH_2, NH), 3050 (CH aromatic), 2968, 2929 (CH aliphatic), 2193 (CN);^{1}H NMR (DMSO-d_6) δ: 1.79 (s, 3H, CH_3), 4.63 (s, 1H, pyran H-4), 6.89 (s, 2H, NH_2, D_2O exchangeable), 7.17–7.38 (m, 4H, Ar-H), 12.11 (s, 1H, NH, D_2O exchangeable) ppm; ^{13}C NMR (DMSO-d_6, 400 MHz): 10.2, 36.1, 57.2, 97.7, 121.1, 128.9, 129.8, 131.7, 136.1, 143.9, 155.2, 161.4 ppm; MS (*m/z*, %): 286 (M^+, 75). *Anal. calcd. for* $C_{14}H_{11}ClN_4O$: C, 58.65; H, 3.87; N, 19.54. Found: C, 58.45; H, 3.91; N, 19.33.

6-Amino-3-methyl-4-(pyridin-3-yl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (8)

To a solution of compound **1** (0.98 g, 0.01 mol), pyridine-3-aldehyde (1.7 g, 0.01 mol) and malononitrile (0.66 g, 0.01 mol) were added. The reaction mixture was heated under reflux for 2 h then left to cool and the formed solid product was collected by filtration and crystallized from ethanol.

Yield: 92%; m.p.: 216-217°C; IR (KBr, cm^{-1}) ν: 3394, 3354 (NH_2, NH), 3066 (CH aromatic), 2985, 2924 (CH aliphatic), 2193 (CN);^{1}H NMR (DMSO-d_6) δ: 1.79 (s, 3H, CH_3), 4.69 (s, 1H, pyran H-4), 6.95 (s, 2H, NH_2, D_2O exchangeable), 7.32–8.46 (m, 4H, pyridine H), 12.15 (s, 1H, NH, D_2O exchangeable) ppm; MS (*m/z*, %): 253

(M^+,11). *Anal. calcd. for* $C_{13}H_{11}N_5O$: C, 61.65; H, 4.38; N, 27.65. Found: C, 61.90; H 4.52; N 27.33.

3.1.2. General procedure for the synthesis of compounds 10a-c

To a solution of compound **1** (0.98 g, 0.01 mol) in ethanol (30 mL) containing triethylamine (1.0 mL) either benzaldehyde (1.08 g, 0.01 mol), 4-methoxybenzaldehyde (1.36 g, 0.01 mol) or 4-chlorobenzaldehyde (1.42 g, 0.01 mol) and 2-aminoprop-1-ene-1,1,3-tricarbonitrile (1.32 g, 0.01mol) was added. The whole reaction mixture, in each case was heated under reflux for 1 h then left to cool then poured onto ice/water mixture containing few drops of hydrochloric acid. The formed solid product, in each case, was collected by filtration and crystallized from ethanol.

5,7-Diamino-3-methyl-4-phenyl-1,4-dihydropyrazolo[4',3':5,6]pyrano[2,3-b]pyridine-6-carbonitrile (**10a**).Yield: 80%; m.p.: >300 °C; IR (KBr, cm^{-1}) ν: 3379, 3213, 2922 ($2NH_2$, NH), 3070 (CH aromatic), 2960, 2922 (CH aliphatic), 2199 (CN);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 4.58 (s, 1H, pyran H-4), 7.10 (s, 2H, NH_2, D_2O exchangeable), 7.06–7.95 (m, 5H, Ar-H), 8.02 (s, 2H, NH_2, D_2O exchangeable), 11.01 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 400 MHz): 14.4, 36.9, 68.3, 91.4, 114.1, 127.1, 128.3, 129.1, 137.3, 144.9, 146.8, 148.4, 150.6, 154.3, 154.9; MS (*m/z*, %): 318 (M^+, 63). *Anal. calcd. for* $C_{17}H_{14}N_6O$: C, 64.14; H, 4.43; N, 26.40. Found: C, 63.90; H, 4.68; N, 26.15.

5,7-Diamino-4-(4-methoxyphenyl)-3-methyl-1,4-dihydropyrazolo-[4',3':5,6]pyrano[2,3-b]pyridine-6-carbonitrile (10b). Yield: 85%; m.p.: 203-205 °C; IR (KBr, cm^{-1}) ν: 3354, 3263, 3130 ($2NH_2$, NH), 3050 (CH aromatic), 2957, 2912 (CH aliphatic), 2206 (CN);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 4.86 (s, 1H, pyran H-4), 6.80 (s, 2H, NH_2, D_2O exchangeable), 7.06–7.95 (m, 4H, Ar-H), 7.95 (s, 2H, NH_2, D_2O exchangeable), 11.01 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 348 (M^+,83.91). *Anal. calcd. for* $C_{18}H_{16}N_6O_2$: C, 62.06; H, 4.63; N, 24.12. Found: C, 62.39; H, 4.71; N, 23.98.

5,7-Diamino-4-(4-chlorophenyl)-3-methyl-1,4-dihydropyrazolo-[4',3':5,6]pyrano[2,3-b]pyridine-6-carbonitrile (10c).Yield: 82%; m.p.: >300°C; IR (KBr, cm^{-1}) ν: 3406, 3290 (NH_2, NH), 3050 (CH aromatic), 2927, 2912 (CH aliphatic), 1681, 1662 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.50 (s, 3H, CH_3), 4.57 (s, 1H,

pyran H-4), 7.15 (s, 2H, NH_2, D_2O exchangeable), 7.17–7.92 (m, 4H, Ar-H), 8.72 (s, 2H, NH_2, D_2O exchangeable), 11. 03 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 353 (M^+, 59). *Anal. calcd. for* $C_{17}H_{13}ClN_6O$: C, 57.88; H, 3.71; N, 23.82. Found: C, 57.58; H, 3.88; N 23.56.

3.1.3. 1-(5-Cyano-4-(4-methoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazol-6-yl)-3-phenylthiourea (12)

To a solution of compound **6b** (2.66 g, 0.01 mol) in dioxane (40 mL) containing triethylamine (1.0 mL), phenylisothiocyanate (1.30 g, 0.01 mol) was added. The reaction mixture was heated under reflux for 2 h. The formed solid product was collected by filtration and crystallized from ethanol. Yield: 90%; m.p.: 192-194 °C; IR (KBr, cm^{-1}) ν: 3360, 3315 (2 NH), 3068 (CH aromatic), 2962, 2926 (CH aliphatic), 2191 (CN), 1170 (C=S);^{1}H NMR (DMSO-d_6) δ: 1.76 (s, 3H, CH_3), 3.72 (s, 3H, OCH3), 4.53 (s, 1H, pyran H- 4), 6.78, 6.80 (2s, 2H, 2NH, D_2O exchangeable), 6.88–7.08 (m, 9H, Ar-H), 12.04 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 417 (M^+,25.15). *Anal. calcd. for* $C_{22}H_{19}N_5O_2S$: C, 63.29; H, 4.59; N, 16.78. Found: C, 63.09; H, 4.68; N, 16.90.

3.1.4. General procedure for synthesis of compounds 13 and 14

To a solution of compound **1** (0.98 g, 0.01 mol) and salicylaldehyde (1.23 g, 0.01mol) in ethanol (30 mL) containing triethylamine (1.0 mL), either malononitrile (0.66 g, 0.01 mol) or ethyl cyanoacetate (1.13 g, 0.01 mol) were added. The whole reaction mixture, in each case, was heated under reflux for 2 h, left to cool then poured onto ice/water mixture containing few drops of hydrochloric acid. The formed solid product, in each case, was collected by filtration and crystallized from ethanol.

5-Amino-1-methyl-3H-chromeno[4',3':4,5]pyrano[2,3-c]pyrazol-6(11bH)-one (13). Yield: 78%; m.p.: >300°C; IR (KBr, cm^{-1}) ν: 3340, 3242 (NH_2, NH), 3050 (CH aromatic), 2999, 2958 (CH aliphatic);^{1}H NMR (DMSO-d_6) δ: 2.48 (s, 3H, CH_3), 4.10 (s, 1H, pyran H), 4.14 (s, 2H, NH_2, D_2O exchangeable), 7.29–7.57 (m, 4H, Ar-H), 11.01 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 400

MHz): 10.6, 26.3, 115.6, 119.1, 125.3, 125.7, 126.0, 134.9, 142.3,152.4, 159.3, 162.0, 162.5, 163.0; MS (*m/z*, %): 269 (M^+, 21). *Anal. calcd. for* $C_{14}H_{11}N_3O_3$: C, 62.45; H, 4.12; N, 15.61. Found: C, 62.39; H 4.18; N 15.88.

1-Methyl-3H-chromeno[4',3':4,5]pyrano[2,3-c]pyrazole-5,6-dione (14). Yield: 82%; m.p.: >300 °C; IR (KBr, cm^{-1}) ν: 3350 (NH), 3050 (CH aromatic), 2927, 2912 (CH aliphatic), 1722 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.48 (s, 3H, CH_3), 6.93-7.60 (m, 4H, Ar-H), 11.01 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 268 (M^+, 29). *Anal. calcd. for* $C_{14}H_8N_2O_4$: C, 62.69; H, 3.01; N, 10.44. Found: C, 62.90; H, 3.20; N, 10.64.

3.1.5. General procedure for synthesis of compounds17a-c

To a solution of compound **1** (0.98 g, 0.01 mol) in ethanol (30 mL) containing triethylamine (1.0 mL), the appropriate aldehyde (0.01mol) and thiourea (0.76 g, 0.01mol) were added. The whole reaction mixture, in each case was heated under reflux for 1 h, left to cool then poured onto ice/water mixture containing few drops of hydrochloric acid. The formed solid product, in each case, was collected by filtration and crystallized from ethanol.

3-Methyl-4-phenyl-1H-pyrazolo[3,4-d]pyrimidine-6(7H)-thione(17a). Yield: 92%; m.p.: 148-150 °C; IR (KBr, cm^{-1}) ν: 3348, 3310 (2 NH), 3050 (CH aromatic), 2949, 2912 (CH aliphatic),1242 (C=S); ^{1}H NMR (DMSO-d_6) δ: 1.76 (s, 3H, CH_3), 3.86 (s, 1H, NH, D_2O exchangeable), 7.12-7.95 (m, 5H, Ar-H), 11.20 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 242 (M^+,12). *Anal. calcd. for* $C_{12}H_{10}N_4S$:C, 59.48; H, 4.16; N, 23.12. Found: C, 59.27; H, 4.19; N, 23.33.

4-(4-Methoxyphenyl)-3-methyl-1H-pyrazolo[3,4-d]pyrimidine-6(7H)-thione (17b). Yield: 85%; m.p.: 154-155 °C; IR (KBr, cm^{-1}) ν: 3367, 3340 (2 NH), 3085 (CH aromatic), 2977, 2914 (CH aliphatic), 1257 (C=S);^{1}H NMR (DMSO-d_6) δ: 1.76 (s, 3H, CH_3), 3.73 (s, 1H, NH, D_2O exchangeable), 3.87 (s, 3H, OCH3), 7.08-8.6 (m, 4H, Ar-H), 11.14 (s, 1H, NH, D_2O exchangeable) ppm;^{13}C NMR (DMSO-d_6, 400 MHz): 13.56, 55.8, 114.9, 127.1, 130.4, 132.3, 136.7, 146.2, 160.9, 162.1; MS (*m/z*, %): 272(M^+, 25). *Anal. calcd. for* $C_{13}H_{12}N_4OS$: C, 57.34; H, 4.44; N, 20.57. Found: C, 57.56; H, 4.58; N, 20.68.

4-(4-Bromophenyl)-3-methyl-1H-pyrazolo[3,4-d]pyrimidine-6(7H)-thione(**17c**).Yield: 89%; mp: 154-155 °C; IR (KBr, cm^{-1}) ν: 3373, 3334 (2 NH), 3085 (CH aromatic), 2977, 2914 (CH aliphatic), 1245 (C=S) ;^{1}H NMR (DMSO-d_6) δ ppm: 2.49 (s, 3H, CH_3), 3.77 (s, 1H, NH, D_2O exchangeable), 7.05-8.52 (m, 4H, Ar-H), 11.25 (s, 1H, NH, D_2O exchangeable); MS (m/z, %): 321(M^+, 18). *Anal. calcd. for* $C_{12}H_9BrN_4S$: C, 44.87; H, 2.82; N, 17.44. Found: C, 44.56; H, 2.62; N, 17.68.

3.1.6. 4-(Ethoxymethylene)-3-methyl-1H-pyrazol-5(4H)-one (19)

A mixture of **1** (0.98 g, 0.01 mol) and triethylorthoformate (1.48 mL, 0.01mol) were heated in an oil bath at 120 ^oC for 30 min then left to cool. The remaining residue was triturated with ethanol and the formed solid product was collected by filtration and crystallized from acetic acid.Yield: 80%; m.p.: >300 °C; IR (KBr, cm^{-1}) ν: 3125 (NH), 2956, 2920 (CH aliphatic), 1678 (C=O);^{1}H NMR (DMSO-d_6) δ: 1.29 (t, 3H, *J* = 7.02 Hz, CH_3), 2.22 (s, 3H, CH_3), 4.15 (q, 2H, *J* = 7.02 Hz, CH_2), 7.38 (s, 1H, CH=C), 12.04 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %):154 (M^+, 20). *Anal. calcd. for* $C_7H_{10}N_2O_2$: C, 54.54; H, 6.54; N, 18.17. Found: C, 54.39; H, 6.88; N, 17.98.

3.1.7. 2-((3-Methyl-5-oxo-1H-pyrazol-4(5H)-ylidene)methyl)malononitrile (20)

A mixture of **1** (0.98 g, 0.01 mol), malonitrile (0.66 g, 0.01mol) , ethyl orthoformate (1.48 mL, 0.01mol) and triethylamine (1 mL) in ethanol (30 mL) was heated under reflux for 2 hr. The reaction mixture was left to cool and the solid product was filtered, dried and cystallized from ethanol.Yield: 80 %; m.p.: >300 °C; IR (KBr, cm^{-1}) ν: 3346 (NH), 2985 (CH aliphatic), 2204, 2179 (2 CN), 1677 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 4.01 (s, 1H, CH), 8.66 (s, 1H, CH=C), 12.04 (s, 1H, NH, D_2O exchangeable); MS (*m/z*, %): 174 (M^+, 32). *Anal. calcd. for* $C_8H_6N_4O$: C, 55.17; H, 3.47; N, 32.17. Found: C, 55.39; H, 3.48; N 32.32.

3.1.8. General procedure for synthesis of compounds 22a-d

To a solution of compound **1** (0.98 g, 0.01 mol) in 1,4-dioxane (30 mL) containing triethylamine (1.0 mL) each of elemental sulfur (0.32 g, 0.01mol) and the appropriate arylisothiocyanate (0.01mol) was added. The whole reaction mixture, in each case was heated under reflux for 3 h, left to cool then poured onto ice/water mixture

containing few drops of hydrochloric acid. The formed solid product was collected by filtration and crystallized from ethanol.

3-Methyl-6-phenyl-1H-pyrazolo[3,4-d]thiazole-5(6H)-thione (22a). Yield: 90%; m.p.: 192-194 °C; IR (KBr, cm^{-1}) ν: 3205 (NH), 3034 (CH aromatic), 2976 (CH aliphatic), 1256 (C=S);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.09-7.50 (m, 5H, Ar-H), 9.75 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 400 MHz): 12.27, 124.5, 128.5,128.9, 129.4, 130.4, 137.8, 139, 180.1; MS (*m/z*, %): 247 (M^+, 18). *Anal. calcd. for* $C_{11}H_9N_3S_2$: C, 53.42; H, 3.67; N, 16.99. Found: C, 53.59; H, 3.88; N, 16.79.

6-(4-Methoxyphenyl)-3-methyl-1H-pyrazolo[3,4-d]thiazole-5(6H)-thione (22b). Yield: 89%;m.p.: 160-162 °C; IR (KBr, cm^{-1}) ν: 3217 (NH), 3020 (CH aromatic), 2976 (CH aliphatic), 1246 (C=S);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 6.87-7.33 (m, 4H, Ar-H), 9.40 (s, 1H, NH, D_2O exchangeable); MS (*m/z*): 277 (M^+, 25). *Anal. calcd. for* $C_{12}H_{11}N_3OS_2$: C, 51.96; H, 4.00; N, 15.15. Found: C, 51.79; H, 3.88; N, 15.30.

6-(4-Chlorophenyl)-3-methyl-1H-pyrazolo[3,4-d]thiazole-5(6H)-thione (22c). Yield: 89%;m.p.: 155-157 °C, IR (KBr, cm^{-1}) ν: 3211 (NH), 3014 (CH aromatic), 2924 (CH aliphatic), 1282 (C=S); ^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.36-7.85 (m, 4H, Ar-H), 9.95 (s, 1H, NH, D_2O exchangeable); MS (*m/z*): 281 (M^+, 40). *Anal. calcd. for* $C_{11}H_8ClN_3S_2$: C, 46.89; H, 2.86; N, 14.91. Found: C, 47.09; H, 2.88; N, 14.79.

6-(4-Bromophenyl)-3-methyl-1H-pyrazolo[3,4-d]thiazole-5(6H)-thione (22d). Yield: 85%;m.p.: 142-144 °C, IR (KBr, cm^{-1}) ν: 3205 (NH), 3012 (CH aromatic), 2976 (CH aliphatic), 1282 (C=S);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.25-7.81 (m, 4H, Ar-H), 9.96 (s, 1H, NH, D_2O exchangeable); MS (*m/z*): 326 (M^+, 28). *Anal. calcd. for* $C_{11}H_8BrN_3S_2$: C, 40.50; H, 2.47; N, 12.88. Found: C, 40.59; H, 2.38; N, 12.79.

3.1.9. General procedure for the synthesis of compounds 25a-c

To a solution of compound 1 (0.98 g, 0.01 mol) in dimethylformamide (40 mL) containing potassium hydroxide (0.40 g, 0.01 mol) phenylisothiocyanate (1.30 g, 0.01

mol) was added. The reaction mixture was stirred at room temperature overnight. To the reaction mixture eitherof 2-bromo-1-phenylethanone (2.0 g, 0.01 mol), 2-bromo-1-(4-chlorophenyl)ethanone (2.35 g, 0.01 mol) or ethyl α-chloroacetate (1.40 g, 0.01 mol) was added and the whole reaction mixture was stirred at room temperature overnight. The solid product, so formed in each case, upon pouring onto ice/water containing hydrochloric acid (till pH 6) was collected by filtration and crystallised from ethanol.

4-(3,4-Diphenylthiazol-2(3H)-ylidene)-3-methyl-1H-pyrazol-5-(4H)-one (25a). Yield: 85%; m.p.: 110-112 °C; IR (KBr, cm^{-1}) ν: 3111 (NH), 3053 (CH aromatic), 2999 (CH aliphatic), 1683 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 7.16 (s, 1H, NH, D_2O exchangeable), 7.25 (s, 1H, H-thiazole), 7.38-7.72 (m, 10H, Ar-H); MS (*m/z*, %): 333 (M^+, 20). *Anal. calcd. for* $C_{19}H_{15}N_3OS$: C, 68.45; H, 4.53; N, 12.60. Found: C, 68.29; H, 4.80; N, 12.79.

4-(3-Phenyl-4-(4-chlorophenyl)thiazol-2(3H)-ylidene)-3-methyl-1H-pyrazol-5-(4H)-one (**25b**). Yield: 82%; m.p.: 182-184 °C; IR (KBr, cm^{-1}) ν: 3120 (NH), 3051 (CH aromatic), 2920 (CH aliphatic), 1699 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 6.98 (s, 1H, thiazole-H), 7.01 (s, 1H, NH, D_2O exchangeable), 7.23-7.67 (m, 9H, Ar-H); ^{13}C NMR (DMSO-d_6, 400 MHz): 32.9, 112.0, 120.4, 121.1, 122.6, 124.3, 129.4, 139.1, 140.4, 154.2, 162.2; MS (*m/z*, %): 367 (M^+, 42). *Anal. calcd. for* $C_{19}H_{14}ClN_3OS$: C, 62.04; H, 3.84; N, 11.42. Found: C, 62.18; H, 3.88; N, 11.58.

4-(4-Hydroxy-3-phenylthiazol-2(3H)-ylidene)-3-methyl-1H-pyrazol-5-(4H)-one (25c).Yield:86%; m.p.: 118-120 °C; IR (KBr, cm^{-1}) ν: 3396 (OH), 3128 (NH), 3026 (CH aromatic), 2920 (CH aliphatic), 1682 (C=O);^{1}H NMR (DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 5.26 (s, 1H, OH, D_2O exchangeable), 7.31 (s, 1H, thiazole-H), 7.38 (s, 1H, NH, D_2O exchangeable), 7.40-7.49 (m, 5H, Ar-H);^{13}C NMR (DMSO-d_6, 400 MHz): 32.6, 112.0, 129.1, 129.2, 134.0, 134.4, 136.0, 164.1, 173.4; MS (*m/z*, %): 373 (M^+, 22). *Anal. calcd. for* $C_{13}H_{11}N_3O_2S$: C, 57.13; H, 4.06; N, 15.37. Found: C, 56.99; H, 4.18; N, 15.58.

3.2. *In vitro* cytotoxic assay

Chemicals: Fetal bovine serum (FBS) and L-glutamine, were purchased from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was purchased from

Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (Saint Louis, USA).

Cell cultures: were obtained from the European Collection of cell Cultures (ECACC, Salisbury, UK) and human gastric cancer (NUGC), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), human breast cancer (MCF), nasopharyngeal carcinoma (HONE1) and normal fibroblast cells (WI38) were kindly provided by the National Cancer Institute (NCI, Cairo, Egypt). They grow as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% heat inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 lg/mL), at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were obtained by plating 1.5 x 10^5 cells/mL for the six human cancer cell lines followed by 24 h of incubation. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (0.5%) of DMSO used in each assay.

3.3. Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was used to predict the toxicity of the synthesized compounds. For the experiment, 4 mg of each compound was dissolved in dimethylsulfoxide (DMSO) and solutions of varying concentrations (10, 100, 1000) μg/mL were obtained by the serial dilution technique using simulated seawater. The solutions were then added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 ml simulated seawater. After 24 h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 s of observation. From this data, the percent of lethality LC_{50} of the brine shrimp nauplii for each concentration and control was calculated.

4. Conclusions

The present research reports the successful synthesis, characterization and evaluation of anticancer activity of pyrazolone, pyranopyrazolone, pyrazolopyrimidine and pyrazolothiazole derivatives. Several compounds showed potent cytotoxic effect with IC_{50} <100 nM. Among these derivatives the pyrazolothiazoles exhibited significant

cytotoxic activity. Compound **22a**showed the maximum cytotoxicity among the tested compounds. Moreover, it was found to be non toxic against shrimp larvae (*Artemiasalina*). Normal fibroblast cells (WI38) were affected to a much lesser extent (IC_{50}>10,000 nM). The obtained results suggest that these compounds may serve as lead chemical entities for further modification in the search of new classes of potential anticancer agents. It could be also concluded that while some of the compounds were not the most potent,their specific activity against particular cell lines makes that of interest for further development as anticancer drugs.

5. References

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