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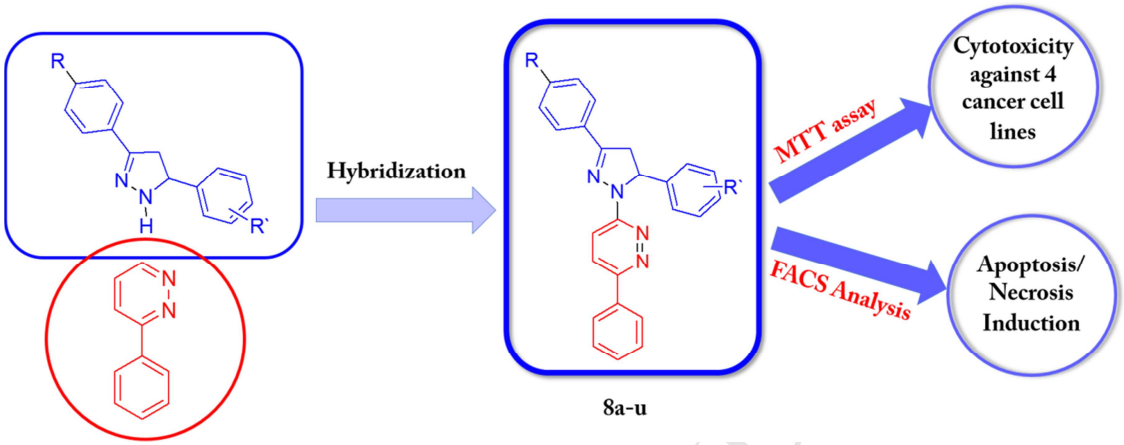

Synthesis and cytotoxic activities of some pyrazoline derivatives bearing phenyl pyridazine core as new apoptosis inducers

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Abstract

The cyclization of chalcones **3a-3u** with 3-hydrazinyl-6-phenylpyridazine **7** under basic condition led to the formation of new pyrazoline derivatives **8a-8u**. All final compounds were characterized by spectral and elemental analyses. They were screened for their antiproliferative activities against A549 (lung), HepG-2 (liver), CaCo-2 (intestinal) and MCF-7 (breast) cancer cell lines. Some of the synthesized compounds exhibited promising antiproliferative activities especially compound **8k** with IC_{50} values of 8.33, 1.67 and 10 μM against HepG-2, MCF-7 and CaCo-2 cancer cell lines, respectively. Moreover, their antiproliferative activity was due to apoptosis rather than necrosis induction except compound **8h** which exhibited equal apoptotic and necrotic properties. Compound **8k** showed 5 fold increase in caspase-3 activity indicating that the apoptosis proceeds via caspase-3 activation.

Keywords: Pyrazolylpyridazines, chalcones, MTT assay, apoptosis, caspase-3.

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1. Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of the abnormal cells [1]. Among all diseases that affect humanity, cancer ranks as the second leading cause of death after cardiovascular diseases [2]. The worldwide cancer burden is expected to increase by as much as 15 million new cancer cases per year by 2020, according to the World Health Organization, unless further preventive measures are put into practice [3]. Generally, cancers of the breast, lung, colorectal, and prostate are the most frequent types in developed countries while those of the stomach, liver, oral cavity, and cervix, in developing countries, although this pattern seems to be evolving, especially due to population aging and life style changes [4,5]. Strategies used for treatment of cancer include surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy [1]. Despite major advances in the chemotherapeutic management of some patients, the use of available chemotherapeutics is often limited mainly due to toxicities and emerging drug resistance [6]. Therefore, there is a continued commitment to discover new anticancer agents.

Pyrazoline is an important scaffold of many bioactive agents that display different biological activities. This can be represented by some pyrazolines that exhibit antiviral [7], antitproliferative [8-15], antibacterial [16-18], anti-inflammatory [9, 16, 19, 20], analgesic [16], and antihyperglycemic activities [21]. Recently, it was reported that 3,5-diaryl pyrazolines **I** and **II (Fig.1)** exhibited potent cytotoxic activity against different cancer cell lines [12, 14]. Furthermore, 6-phenylpyridazine can be considered as an essential core responsible for various biological activities of different candidates. In literature, some pyridazines act as antidepressant [22], antinociceptive [23], interleukin-1β inhibitory [24], formyl peptide receptors agonists [25], antidiabetic [26], antimicrobial [27] and antiproliferative agents [27-33]. 6-Arylpyridazines **III** and **IV (Fig. 1)** revealed promising cytotoxic activity [29, 32].

From the molecular design point of view, the combination of two pharmacophores into a single molecule represents one of the methods that can be adopted for the synthesis of new anticancer molecules [34-40]. This approach was supported by compound **V** which is a pyrazole substituted pyridazinone with potent antiproliferative and c-Met inhibitory activities [41]. Therefore, based on the previous findings, this work deals with

synthesis of a novel series **8a-u** through hybridization of the pharmacophores, 3,5-diarylpyrazoline and 6-phenylpyridazine **(Fig. 2)**, hoping to obtain new promising antiproliferative hits. Additionally, all the synthesized derivatives were screened against diverse human tumor cell lines, including A549 “lung”, HEPG2 “liver”, MCF7 “breast” and CaCo2 “intestinal” cancer cell lines. Moreover, the most active compounds were further tested for their necrotic and/or apoptotic properties. Additionally, the apoptotic mechanism of compound **8k** having the most promising IC_{50} was investigated through caspase-3 colorimetric assay.

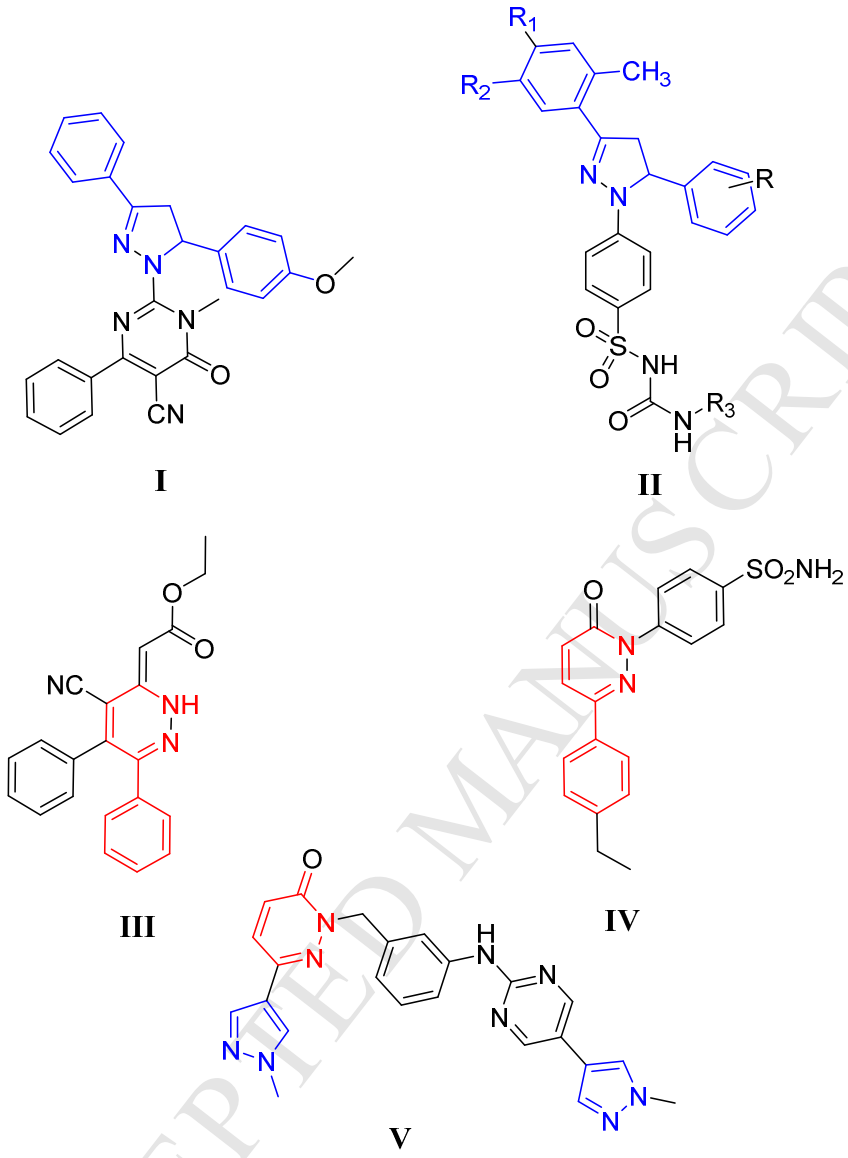


Fig. 1. Structures of some 3,5-diarylpyrazolines, 6-arylpyridazines and pyrazole-pyridazine hybrid with antiproliferative activity

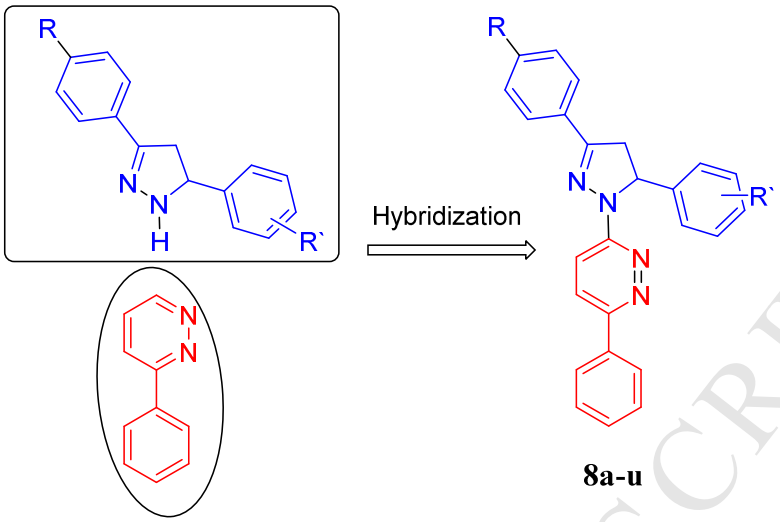


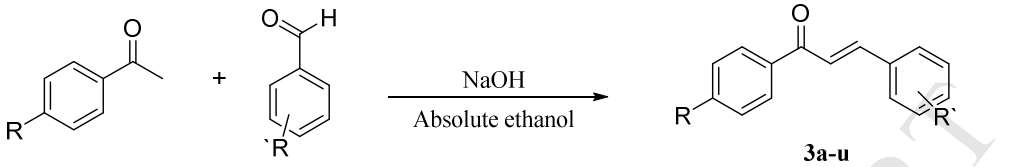
Fig.2. General structure of target compounds

2. Results and discussion

2.1. Chemistry

The target compounds **8a–8u** were prepared as outlined in Scheme 3. First, a series of chalcones **3a-u** were prepared according to scheme 1 by the Claisen–Schmidt condensation between acetophenones **1a-c** and different aromatic aldehydes **2a-g** in absolute ethanol in the presence of sodium hydroxide in 80–85% yield [42-53]. Furthermore, the reaction of acetophenone **1a** with glyoxilic acid **4** was followed by treatment with hydrazine hydrate to afford 6-phenyl-3-pyridazinone **5** [54]. Moreover, reflux of **5** in phosphorous oxychloride resulted in 3-chloropyridazine **6** that was reacted with hydrazine hydrate in absolute ethanol to obtain **7** (Scheme 2) [55]. The target pyrazoline derivatives **8a-u** were prepared by reaction of 3-hydrazinyl-6-phenylpyridazine **7** with the appropriate chalcones **3a-u** in absolute ethanol in the presence of sodium hydroxide (Scheme 3). All the final compounds **8a-u** were characterized by IR, ^{1}H NMR and elemental analyses. ^{1}H NMR spectra showed three signals doublet of doublet at 3.05-3.32, 3.77-3.94 and 5.83-6.17 ppm corresponding to the pyrazoline protons in addition to the signals of pyridazine and aromatic protons (c.f. experimental part). On the other hand, ^{13}C NMR of **8u** as a representative example

revealed signals at 43.4 and 62.1 of CH_2 and CH of the pyrazoline ring, respectively, as well as signals at 55.4, 56.1 and 60.7 attributed to four methoxy moieties.



1a-c

1a R= H
1b R= Cl
1c R= OCH_3

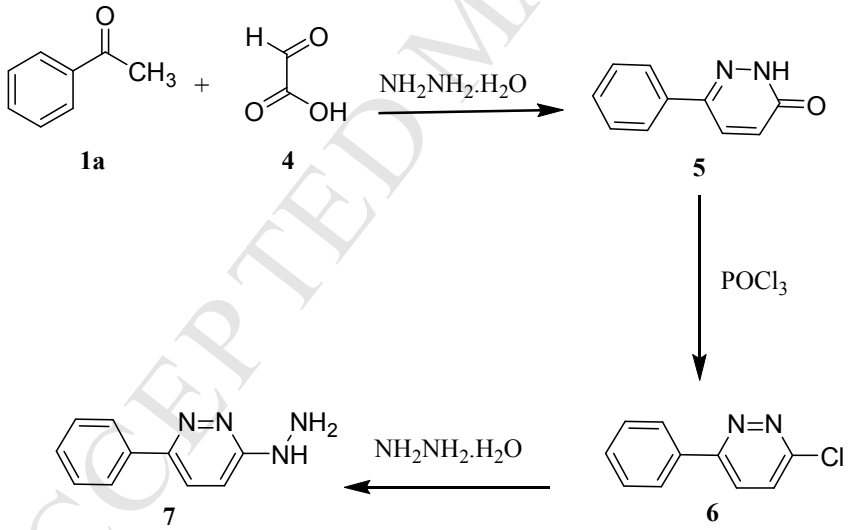
2a-g

2a R`=H
2b R`= 4-Cl
2c R`= 4-F
2d R`= 4-CF_3
2e R`= 4-OCH_3
2f R`= 2,4-$(OCH_3)_2$
2g R`= 3,4,5-$(OCH_3)_3$

3a-u

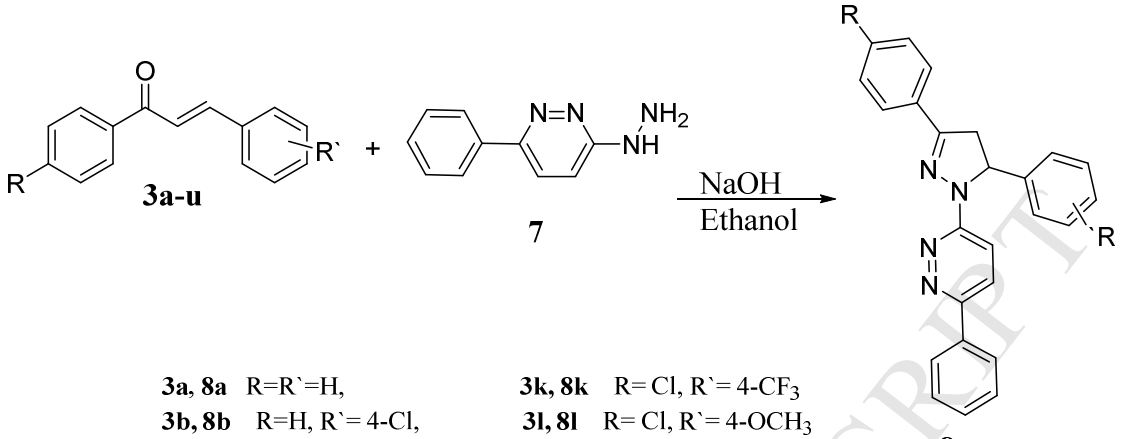
3a R=R`=H,
3b R=H, R`= 4-Cl,
3c R=H, R`= 4-F,
3d R=H, R`= 4-CF_3,
3e R=H, R`= 4-OCH_3,
3f R=H, R`= 2,4-$(OCH_3)_2$,
3g R=H, R`= 3,4,5-$(OCH_3)_3$,
3h R= Cl, R`= H,
3i R= Cl, R`= 4-Cl,
3j R= Cl, R`= 4-F,
3k R= Cl, R`= 4-CF_3
3l R= Cl, R`= 4-OCH_3
3m R= Cl, R`= 2,4-$(OCH_3)_2$
3n R= Cl, R`= 3,4,5-$(OCH_3)_3$
3o R= OCH_3, R`= H
3p R= OCH_3, R`= 4-Cl
3q R= OCH_3, R`= 4-F
3r R= OCH_3, R`= 4-CF_3
3s R= OCH_3, R`= 4-OCH_3
3t R= OCH_3, R`= 2,4-$(OCH_3)_2$
3u R= OCH_3, R`= 3,4,5-$(OCH_3)_3$

Scheme 1. Preparation of chalcones **3a-u**



1a **4** **5** **6** **7**

Scheme 2. Preparation of 3-hydrazinyl-6-phenylpyridazine **7**



3a, 8a R=R`=H, **3k, 8k** R= Cl, R`= 4-CF_3
3b, 8b R=H, R`= 4-Cl, **3l, 8l** R= Cl, R`= 4-OCH_3
3c, 8c R=H, R`= 4-F, **3m, 8m** R= Cl, R`= 2,4-$(OCH_3)_2$
3d, 8d R=H, R`= 4-$CF_3$, **3n, 8n** R= Cl, R`= 3,4,5-$(OCH_3)_3$
3e, 8e R=H, R`= 4-$OCH_3$, **3o, 8o** R= $OCH_3$, R`= H
3f, 8f R=H, R`= 2,4-$(OCH_3)_2$, **3p, 8p** R= $OCH_3$, R`= 4-Cl
3g, 8g R=H, R`= 3,4,5-$(OCH_3)_3$, **3q, 8q** R= $OCH_3$, R`= 4-F
3h, 8h R= Cl, R`= H, **3r, 8r** R= $OCH_3$, R`= 4-CF_3
3i, 8i R= Cl, R`= 4-Cl, **3s, 8s** R= $OCH_3$, R`= 4-OCH_3
3j, 8j R= Cl, R`= 4-F, **3t, 8t** R= $OCH_3$, R`= 2,4-$(OCH_3)_2$
3u, 8u R= OCH_3, R`= 3,4,5-$(OCH_3)_3$

Scheme 3. Preparation of target compounds **8a-u**

2.2. Biological evaluation

2.2.1. Antiproliferative *activity*

In-vitro antiproliferative activity of the tested compounds was performed utilizing MTT cell viability assay [56]. The target compounds **8a-u** were screened against A549 "lung", HEPG2 "liver", MCF7 "breast" and CaCo2 "intestinal" cancer cell lines, Doxorubicin was used as a reference standard. From the observed data **(Table 1)**, it has been noticed that many compounds exerted significant activity against HepG-2 "liver" cell line compared with the other assayed cell lines. Considering the observed antiproliferative screening data against A549 "lung" cancer cell line, only compounds **8d** and **8f** revealed the highest activities with IC_{50} values 9.16 and 9.56 μM, respectively. However, compounds **8c** and **8k** resulted in 50% viability of CaCo-2 "intestinal" cancer cell line at concentration of 10 μM. Additionally, compound **8q** exerted moderate activity with IC_{50} value 18.07 μM, while, compounds **8a**, **8d** and **8r** revealed mild activity with

IC_{50} values 40.86 - 65.00 μM against the same cell line. Otherwise, compounds **8k**, **8j**, **8i** and **8h** exhibited promising activity against MCF-7 "breast" cancer cell line with IC_{50} values 1.67, 3.75, 8.80 and 10 μM, respectively. On the other hand, compounds **8a** and **8q** revealed moderate activity, meanwhile, compounds **8p** and **8r** showed mild activity.

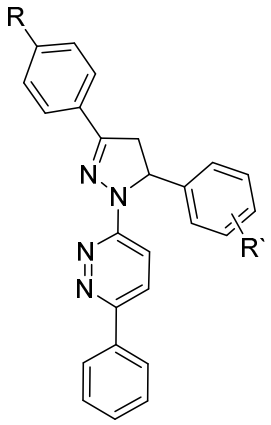
Furthermore, compounds **8b-d**, **8h-k** and **8q** resulted in promising activity against HepG-2 "liver" cancer cell line with IC_{50} values 6.31-10 μM. Structure activity relationship study of the obtained antiproliferative activity of the target compounds **8a-u** against HepG-2 cancer cell line indicated that substitution with electron withdrawing groups at one or both phenyl rings of the pyrazoline moiety, seemed more favorable for activity than the case when electron donating groups were incorporated in either phenyl rings. However, compound **8q** is an exception (IC_{50}= 9.16 μM).

Moreover, the most active compounds **8b**, **8c**, **8d**, **8f**, **8h**, **8i**, **8j** and **8k** were screened for their cytotoxicity against human embryonic kidney cells 293 as example of normal cells at 10, 100, 200 and 300 μM. The % viable cells and IC_{50} values were shown in Table 2. All tested compounds were safe to the tested normal cells up to 300 μM. Therefore, these compounds were selective to the tested cancer cell lines.

2.2. 2. Evaluation of Cell Death Pathways

The most active compounds were further screened at 10 μM for their cytotoxicity mechanism either apoptosis or necrosis by applying the flow cytometry technique. The obtained results were shown in the flow cytometry charts (Figures 3-6). The % apoptosis or necrosis induction for each compound was calculated by taking the difference between % apoptotic or necrotic cells of each compound and that of the negative control, and they were summarized in Table 3. From the obtained data, it can be concluded that all tested compounds can be considered as apoptotic inducers rather than necrotic inducers except compound **8h** which exhibited equal apoptotic and necrotic properties.

Table 1: IC_{50} of the target compounds **8a-u** against the tested cancer cell lines in MTT assay



8a-u

Compound ID	R	R`	IC50 (μM) against			
			A549	**HepG2**	**MCF7**	**CaCo2**
8a	H	H	>100	10	23.75	40.86
8b	H	4-Cl	100	6.31	100	100
8c	H	4-F	89.2	8.42	100	10
8d	H	4-CF_3	9.16	10	100	50
8e	H	4-OCH_3	91.67	>100	100	>100
8f	H	2,4-$(OCH_3)_2$	9.56	100	>100	>100
8g	H	3,4,5-$(OCH_3)_3$	83.75	100	70	>100
8h	Cl	H	96.67	8.75	10	100
8i	Cl	4-Cl	>100	10	8.80	>100
8j	Cl	4-F	>100	10	3.75	>100
8k	Cl	4-CF_3	>100	8.33	1.67	10
8l	Cl	4-OCH_3	100	>100	100	>100
8m	Cl	2,4-$(OCH_3)_2$	>100	>100	>100	>100
8n	Cl	3,4,5-$(OCH_3)_3$	83.33	>100	100	>100
8o	OCH_3	H	75.90	>100	>100	>100
8p	OCH_3	4-Cl	100	>100	58	>100
8q	OCH_3	4-F	>100	9.16	20	18.07
8r	OCH_3	4-CF_3	>100	>100	46.8	65
8s	OCH_3	4-OCH_3	>100	>100	100	>100
8t	OCH_3	2,4-$(OCH_3)_2$	100	>100	100	>100
8u	OCH_3	3,4,5-$(OCH_3)_3$	>100	>100	100	>100
Doxorubicin	---------	----------	2.12	3.82	2.78	3.00

Table 2: % viable cells and IC_{50} of the most active compounds against normal human embryonic kidney cells 293

Compd. ID	% Viability of normal human embryonic kidney 293± SE at 10 µM	100 µM	200 µM	300 µM	IC_{50} (µM)
8b	96.63 ± 3.13	91.17 ± 2.58	88.33 ± 1.26	74.03 ± 1.64	> 300
8c	93.68 ± 4.26	91.88 ± 5.41	86.31 ± 2.46	78.61 ± 4.91	> 300
8d	89.75 ± 1.96	86.25 ± 1.79	82.32 ± 1.61	70.75 ± 5.24	> 300
8f	85.16 ± 4.62	82.87 ± 4.50	81.12 ± 0.76	74.19 ± 1.47	> 300
8h	83.96 ± 2.33	81.56 ± 1.64	78.12 ± 2.13	71.57 ± 2.46	> 300
8i	85.60 ± 2.08	81.67 ± 3.60	79.81 ± 2.62	76.42 ± 1.94	> 300
8j	85.93 ± 4.30	82.32 ± 1.79	75.77 ± 2.02	68.78 ± 4.45	> 300
8k	92.70 ± 5.57	87.67 ± 0.93	84.62 ± 3.45	79.10 ± 1.80	> 300

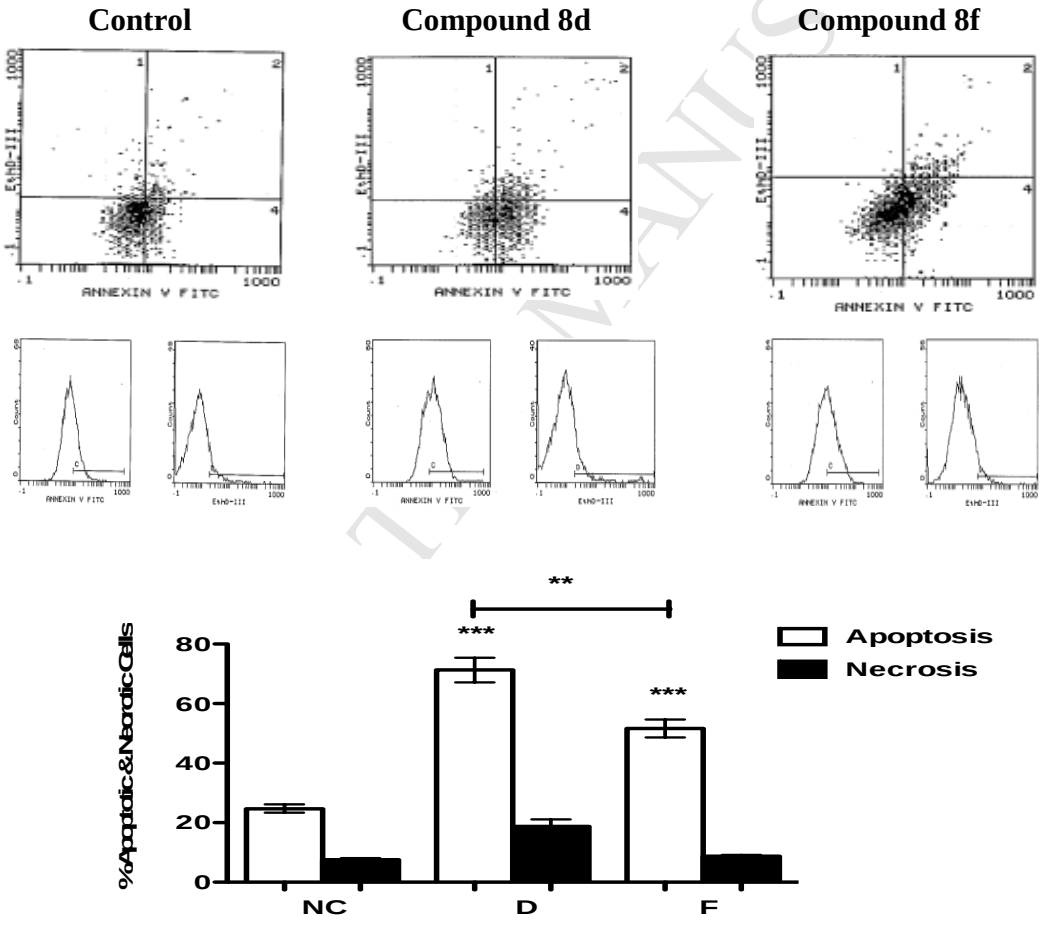


Figure 3. Effect of compound **8d**, **8f** on the induction of apoptosis/necrosis in A549 cells.

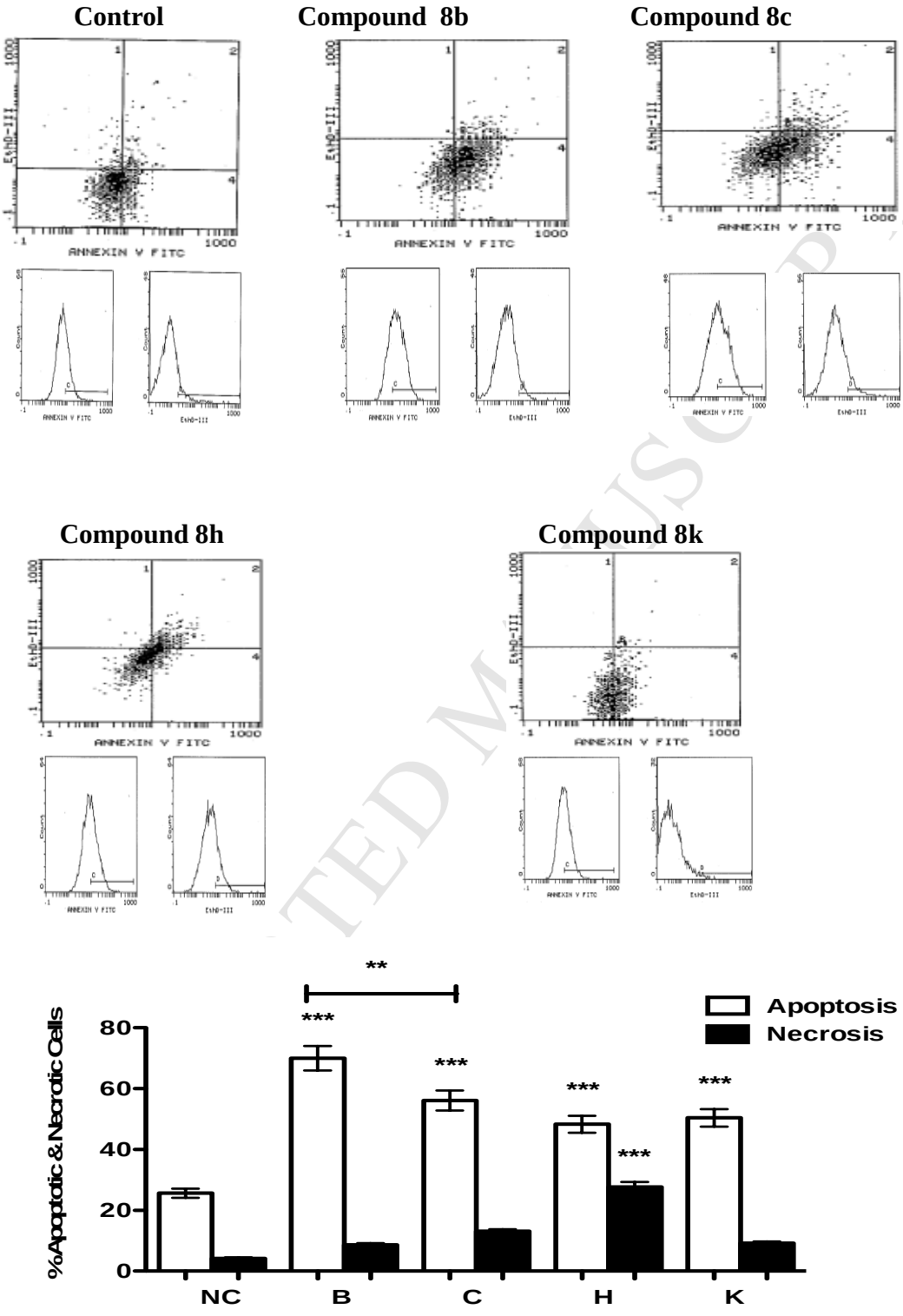


Figure 4. Effect of compound **8b**, **8c**, **8h** and **8k** on the induction of apoptosis/necrosis in HepG2 cells.

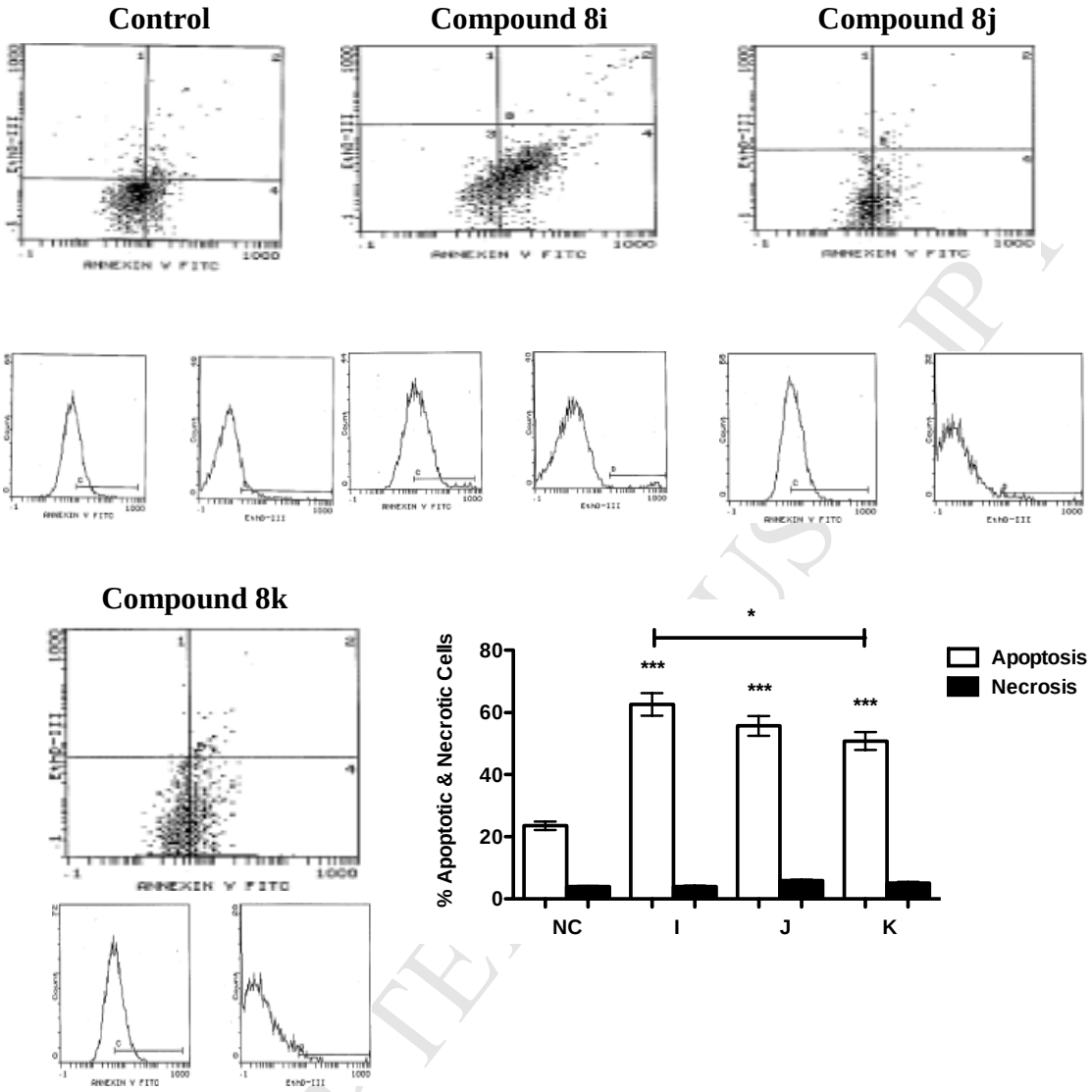


Figure 5. Effect of compound **8i**, **8j** and **8k** on the induction of apoptosis/necrosis in MCF-7 cells.

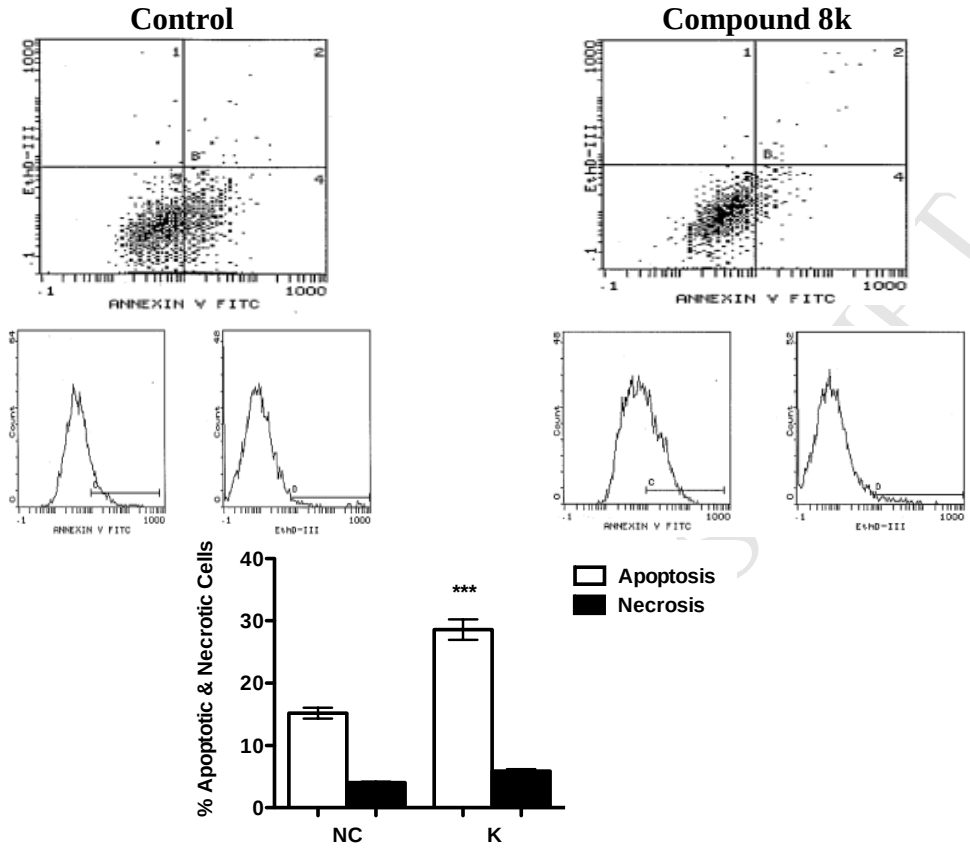


Figure 6. Effect of compound **8k** on the induction of apoptosis/necrosis in CaCo2 cells.

Table 3: % apoptotic and necrotic induction of the most active compounds at 10 μM

Compd. ID	A549		HepG-2		MCF7		CaCo2	
	%Apoptotic induction	% necrotic induction	%Apoptotic induction	% necrotic induction	%Apoptotic induction	% necrotic induction	%Apoptotic induction	% necrotic induction
8b	NT	NT	44.40	4.40	NT	NT	NT	NT
8c	NT	NT	30.50	8.84	NT	NT	NT	NT
8d	46.60	11.11	NT	NT	NT	NT	NT	NT
8f	27.00	1.04	NT	NT	NT	NT	NT	NT
8h	NT	NT	22.70	23.45	NT	NT	NT	NT
8i	NT	NT	NT	NT	39.00	0.10	NT	NT
8j	NT	NT	NT	NT	32.10	1.98	NT	NT
8k	NT	NT	24.80	4.97	27.20	1.26	13.40	1.87

* NT: not tested

2.2.3. Caspase-3 activation assay

Caspase-3 is a ubiquitously-expressed cysteine protease involved in programmed cell death (apoptosis). In non-apoptotic cells, this enzyme exists as an inactive zymogen known as procaspase-3. Intracellular procaspase-3 is activated when cells are committed to die via apoptosis. The active enzyme cleaves a number of intracellular protein targets important for cell survival. Therefore, monitoring caspase-3 enzyme activity is a fundamental means to measure apoptosis in cells. In an attempt to find out whether the induction of apoptosis of compounds **8b**, **8c**, **8d**, **8f**, **8i**, **8j** and **8k** is dependent on caspase-3 activity or not, **8k** was selected as a representative for testing caspase-3 activity in MCF-7 cell lines as it is the most active candidate with promising IC_{50} = 1.67 μM. Caspase-3 colorimetric detection kit was used. The cleavage of the caspase 3-specific tetrapeptide substrate (ac-Asp-Glu-Val-Asp-pNA) that is labeled with the chromophore p-nitroaniline (pNA) by the activated caspase-3 leads to the release of the chromophore pNA. The free pNA group has a yellow color which is visible in the wells and was quantitated spectrophotometrically at 405 nm. A significant 5-fold increase was observed in the induction of caspase-3 in MCF-7 cells treated with compound **8k** relative to the control (Fig. 7). Therefore, the apoptotic pathway may proceed through caspase-3 activation.

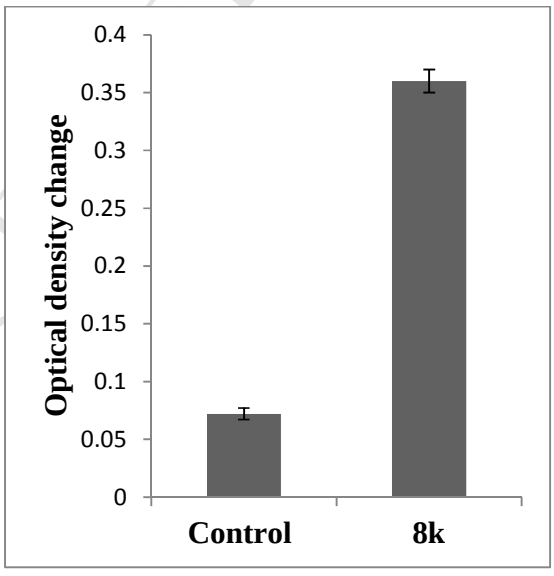


Figure 7. Effect of compound **8k** on caspase-3 activity in MCF-7 cell lines compared to the negative control (untreated cells).

3. Conclusion

In summary, novel pyrazolyl pyridazine derivatives **8a-8u** were synthesized by the cyclization of chalcones **3a-3u** with 3-hydrazinyl-6-phenylpyridazine **7** under basic condition. All target compounds **8a-u** were screened for their antiproliferative activities against A549 (lung), HepG-2 (liver), CaCo-2 (intestinal) and MCF-7 (breast) cancer cell lines using MTT cell viability assay. Many compounds exhibited moderate to good activity against MCF-7 and HepG-2 cancer cell lines. On the other hand, only compounds **8d** and **8f** showed promising activity against A549 cancer cell lines, whereas, compounds **8c** and **8k** exhibited good activity against CaCo-2 cell lines. Moreover, these compounds can be considered as apoptotic inducers except compound **8h** which showed equal apoptotic and necrotic properties. They may exhibit their apoptosis through caspase-3 activation as illustrated by compound **8k** that resulted in 5 fold increase in caspase-3 activity in MCF-7 cell lines.

4. Experimental

4.1. Chemistry

Melting points were recorded on Stuart SMP10 digital melting point apparatus. IR spectra (KBr disc) were recorded on a Shimadzu FT-IR 8400S infrared spectrophotometer. NMR spectra were recorded on a Bruker Ascend 400/ R (^{1}H: 400, ^{13}C: 100 MHz) spectrometer. Elemental analyses were carried out at the Regional center for mycology and biotechnology, Al-Azhar University, Egypt. Chalcones **3a-u** [42-53] and 6-phenylpyridazines **5-7** [54, 55] were prepared according to the reported procedures.

4.1.1. General procedure for preparation of **8a-u**

A mixture of the appropriate chalcone **3a-u** (1.5 mmol), the hydrazine derivative **7** (1.5 mmol) and sodium hydroxide (3.75 mmol) in absolute ethanol (5 ml) was heated under reflux for 12 hours. The obtained precipitate on cooling was filtered and washed with water and crystallized from ethanol.

4.1.1.1. 3-(3,5-Diphenyl-4,5-dihydropyrazol-1-yl)-6-phenylpyridazine **8a**

Obtained from the reaction of **3a** with **7**, mp 198-199˚C, yield 64% (0.36 g). IR: ν_{max}/cm^{-1} 3061, 3030, 2916, 1589, 1547, 1458, 1435. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.23 (dd, 1H, *J* = 5.26, 17.42 Hz, pyrazoline proton), 3.84 (dd, 1H, *J* = 12.20, 17.40 Hz, pyrazoline proton), 5.94 (dd, 1H, *J* = 5.22, 12.14 Hz, pyrazoline proton), 7.11-7.23 (m, 3H, arom. protons), 7.27-7.38 (m, 8H, arom. protons), 7.64 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.71 (d, 2H, *J* = 6.68 Hz, arom. protons), 7.76 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.89 (d, 2H, *J* = 7.44 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 42.9 (pyrazoline CH_2), 61.9 (pyrazoline CH), 115.2, 125.3, 126.0, 126.1, 126.2, 127.4, 128.7, 128.8, 129.6, 132.0, 136.8, 142.3, 151.5, 152.4, 155.6 (aromatic carbons).Anal.Calcd. for $C_{25}H_{20}N_4$ (376.46): C, 79.76; H, 5.35; N, 14.88. Found: C, 79.92; H, 5.41; N, 15.03.

4.1.1.2. 3-[5-(4-Chlorophenyl)-3-phenyl-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8b**

Obtained from the reaction of **3b** with **7**, mp 225-227˚C, yield 50% (0.31 g). IR: ν_{max}/ cm^{-1} 3048, 3028, 2924, 1591, 1553, 1468, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.29 (dd, 1H, *J* = 5.48, 17.44 Hz, pyrazoline proton), 3.94 (dd, 1H, *J* = 12.20, 17.44 Hz, pyrazoline proton), 6.00 (dd, 1H, *J* = 5.46 , 12.18 Hz, pyrazoline proton), 7.27-7.34 (m, 5H, arom. protons), 7.41- 7.49 (m, 5H, arom. protons), 7.75 (d, 1H, *J* = 9.36 Hz, pyridazine proton), 7.80 (dd, 2H, *J* = 1.66, 7.82 Hz, arom. protons), 7.85 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.99 (d, 2H, *J* = 7.08 Hz, arom. protons). Anal.Calcd. for $C_{25}H_{19}ClN_4$ (410.91): C, 73.08; H, 4.66; N, 13.63. Found: C, 73.17; H, 4.69; N, 13.78.

4.1.1.3. 3-[5-(4-Fluorophenyl)-3-phenyl-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8c**

Obtained from the reaction of **3c** with **7**, mp 166-168˚C, yield 41% (0.24 g). IR: ν_{max}/cm^{-1} 3061, 2935, 1591, 1553, 1506, 1470, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.31 (dd, 1H, *J* = 5.36, 17.44 Hz, pyrazoline proton), 3.94 (dd, 1H, *J* = 12.16, 17.44 Hz, pyrazoline proton), 6.02 (dd, 1H, *J* = 5.32, 12.16 Hz, pyrazoline proton), 7.00 (t, 2H, *J* = 8.70 Hz, arom. protons), 7.35- 7.49 (m, 8H, arom. protons), 7.75 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.81 (dd, 2H, *J* = 1.62, 7.90 Hz, arom. protons), 7.85 (d, 1H, *J* = 9.40 Hz,

pyridazine proton), 7.99 (d, 2H, *J* = 7.04 Hz, arom. protons). Anal.Calcd. for $C_{25}H_{19}FN_4$ (394.46): C, 76.12; H, 4.86; N, 14.20. Found: C, 76.28; H, 4.89; N, 14.37.

4.1.1.4. 3-Phenyl-6-{3-phenyl-5-[4-(trifluoromethyl)phenyl]-4,5-dihydropyrazol-1-yl}pyridazine **8d**

Obtained from the reaction of **3d** with **7**, mp 225-227˚C, yield 60% (0.40 g). IR: ν_{max}/ cm^{-1} 3055, 2950, 2849, 1591, 1553, 1470, 1441. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.20 (dd, 1H, *J* = 5.64, 17.44 Hz, pyrazoline proton), 3.88 (dd, 1H, *J* = 12.28, 17.44 Hz, pyrazoline proton), 5.97 (dd, 1H, *J* = 5.62, 12.22 Hz, pyrazoline proton), 7.30-7.39 (m, 8H, arom. protons), 7.49 (d, 2H, *J* = 8.20 Hz, arom. protons), 7.68 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.70 (dd, 2H, *J* = 1.80, 7.72 Hz, arom. protons), 7.78 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.89 (d, 2H, *J* = 7.08 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 42.8 (pyrazoline CH_2), 61.6 (pyrazoline CH), 115.3, 122.7, 125.4, 125.5, 125.8, 125.9, 126.4, 128.7, 128.8, 128.9, 130.3, 131.6, 136.6, 146.3, 151.4, 152.9, 155.5 (aromatic carbons). Anal.Calcd. for $C_{26}H_{19}F_3N_4$ (444.45): C, 70.26; H, 4.31; N, 12.61. Found: C, 70.44; H, 4.37; N, 12.80.

4.1.1.5. 3-[5-(4-Methoxyphenyl)-3-phenyl-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8e**

Obtained from the reaction of **3e** with **7**, mp 173-175˚C, yield 66% (0.40 g). IR: ν_{max}/cm^{-1} 3049, 3011, 2955, 2930, 2835, 1587, 1549, 1508, 1470, 1456, 1435. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.32 (dd, 1H, *J* = 5.18, 17.42 Hz, pyrazoline proton), 3.77 (s, 3H, OCH_3), 3.94 (dd, 1H, *J* = 12.06, 17.42 Hz, pyrazoline proton), 6.00 (dd, 1H, *J* = 5.14, 12.06 Hz, pyrazoline proton), 6.84 (d, 2H, *J* = 8.64 Hz, arom. protons), 7.33 (d, 2H, *J* = 8.68 Hz, arom. protons), 7.38- 7.48 (m, 6H, arom. protons), 7.72 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.80-7.85 (m, 3H, 2H arom. protons + 1H pyridazine proton), 7.99 (d, 2H, *J* = 7.28 Hz, arom. protons). Anal.Calcd. for $C_{26}H_{22}N_4O$ (406.49): C, 76.83; H, 5.46; N, 13.78. Found: C, 76.98; H, 5.53; N, 13.96.

4.1.1.6. 3-[5-(2,4-Dimethoxyphenyl)-3-phenyl-4,5-dihydropyrazol-1-yl]-6-phenyl-pyridazine **8f**

Obtained from the reaction of **3f** with **7**, mp 185-187˚C, yield 69% (0.45 g). IR: ν_{max}/cm^{-1} 3050, 2928, 2890, 1589, 1551, 1466, 1437. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.09 (dd, 1H, *J* = 5.00, 17.36 Hz, pyrazoline proton), 3.66 (s, 3H, OCH_3), 3.74-3.81 (m, 4H, OCH_3 + pyrazoline proton), 6.10 (dd, 1H, *J*= 4.98, 11.98 Hz, pyrazoline proton), 6.24 (dd, 1H, *J* = 2.24, 8.40 Hz, arom. proton), 6.40 (d, 1H, *J* = 2.24 Hz, arom. proton), 6.90 (d, 1H, *J* = 8.44 Hz, arom. proton), 7.28-7.38 (m, 6H, arom. protons), 7.65 (d, 1H, *J* = 9.44 Hz, pyridazine proton), 7.69 (d, 2H, *J* = 6.52 Hz, arom. protons), 7.77 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.91 (d, 2H, *J* = 7.24 Hz, arom. protons). Anal.Calcd. for $C_{27}H_{24}N_4O_2$ (436.51): C, 74.29; H, 5.54; N, 12.84. Found: C, 74.52; H, 5.57; N, 13.03.

4.1.1.7. 3-Phenyl-6-[3-phenyl-5-(3,4,5-trimethoxyphenyl)-4,5-dihydropyrazol-1-yl] pyridazine **8g**

Obtained from the reaction of **3g** with **7**, mp 183-185˚C, yield 46% (0.32 g). IR: ν_{max}/cm^{-1} 3051, 2968, 2833, 1589, 1549, 1458, 1435. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.21 (dd, 1H, *J* = 5.56, 17.44 Hz, pyrazoline proton), 3.71 (s, 3H, OCH_3), 3.72 (s, 6H, 2 OCH_3), 3.82 (dd, 1H, *J* = 12.20, 17.44 Hz, pyrazoline proton), 5.86 (dd, 1H, *J* = 5.58, 12.14 Hz, pyrazoline proton), 6.47 (s, 2H, arom. protons), 7.30-7.40 (m, 6H, arom. protons), 7.68 (d, 1H, *J* = 9.48 Hz, pyridazine proton), 7.71 (dd, 2H, *J* = 1.70, 7.90 Hz, arom. protons), 7.79 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.92 (d, 2H, *J* = 7.20 Hz, arom. protons). Anal.Calcd. for $C_{28}H_{26}N_4O_3$ (466.53): C, 72.09; H, 5.62; N, 12.01. Found: C, 72.26; H, 5.69; N, 12.17.

4.1.1.8. 3-[3-(4-Chlorophenyl)-5-phenyl-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8h**

Obtained from the reaction of **3h** with **7**, mp 215-217˚C, yield 42% (0.26 g). IR: ν_{max}/ cm^{-1} 3075, 3057, 3028, 2918, 2849, 1593, 1545, 1493, 1462, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.30 (dd, 1H, *J*= 5.32, 17.36 Hz, pyrazoline proton), 3.91 (dd, 1H, *J* = 12.26, 17.38 Hz, pyrazoline proton), 6.06 (dd, 1H, *J* = 5.32, 12.20 Hz, pyrazoline proton), 7.24-7.48 (m, 10H, arom. protons), 7.73 (d, 2H, J = 8.44 Hz, arom. protons), 7.75 (d, 1H, J = 9.32 Hz, pyridazine proton), 7.84 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.99 (d, 2H, *J*

= 7.16 Hz, arom. protons). Anal.Calcd. for $C_{25}H_{19}ClN_4$ (410.91): C, 73.08; H, 4.66; N, 13.63. Found: C, 73.22; H, 4.71; N, 13.81.

4.1.1.9. 3-[3,5-bis(4-Chlorophenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8i**

Obtained from the reaction of **3i** with **7**, mp 189-191˚C, yield 46% (0.31 g). IR: ν_{max}/ cm^{-1} 3084, 3032, 2922, 2853, 1591, 1551, 1464, 1441. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.26 (dd, 1H, *J*= 5.56, 17.44 Hz, pyrazoline proton), 3.91 (dd, 1H, *J* = 12.26, 17.42 Hz, pyrazoline proton), 6.01 (dd, 1H, *J* = 5.54, 12.22 Hz, pyrazoline proton), 7.27-7.33 (m, 3H, arom. protons), 7.39-7.49 (m, 6H, arom. protons), 7.72 (d, 2H, J = 8.56 Hz, arom. Protons), 7.76 (d, 1H, J = 9.40 Hz, pyridazine proton), 7.83 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.99 (d, 2H, *J* = 7.08 Hz, arom. protons). Anal.Calcd. for $C_{25}H_{18}Cl_2N_4$ (445.36): C, 67.42; H, 4.07; N, 12.58. Found: C, 67.53; H, 4.10; N, 12.73.

4.1.1.10. 3-[3-(4-Chlorophenyl)-5-(4-fluorophenyl)-4,5-dihydropyrazol-1-yl]-6-phenyl-pyridazine **8j**

Obtained from the reaction of **3j** with **7**, mp 203-205˚C, yield 63% (0.40 g). IR: ν_{max}/cm^{-1} 3075, 3053, 3036, 2922, 2853, 1603, 1541, 1514, 1460, 1441. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.15 (dd, 1H, *J* = 5.42, 17.42 Hz, pyrazoline proton), 3.78 (dd, 1H, *J* = 12.24, 17.40 Hz, pyrazoline proton), 5.91 (dd, 1H, J = 5.38, 12.18 Hz, pyrazoline proton), 6.89 (t, 2H, J = 8.64 Hz, arom. protons), 7.24 (dd, 2H, J= 5.34, 8.50 Hz, arom. protons), 7.30–7.37 (m, 5H, arom. protons), 7.60-7.65 (m, 3H, 2H arom. protons + pyridazine proton), 7.71 (d, 1H, *J* = 9.36 Hz, pyridazine proton), 7.88 (d, 2H, *J* = 7.24 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 42.8 (pyrazoline CH_2), 61.5 (pyrazoline CH), 115.2, 115.6, 115.8, 125.4, 126.2, 127.1, 127.4, 127.7,127.8, 128.7, 128.8, 128.9, 129.0, 130.2, 130.4, 135.6, 136.7, 137.9, 138.0, 150.3, 152.8, 155.5, 160.9, 163.3 (aromatic carbons). Anal.Calcd. for $C_{25}H_{18}ClFN_4$ (428.90): C, 70.01; H, 4.23; N, 13.06. Found: C, 70.19; H, 4.29; N, 13.22.

4.1.1.11. 3-{3-[4-Chlorophenyl]-5-[4-(trifluoromethyl)phenyl]-4,5-dihydropyrazol-1-yl}-6-phenylpyridazine **8k**

Obtained from the reaction of **3k** with **7**, mp 201-203˚C, yield 49% (0.35 g). IR: ν_{max}/ cm^{-1} 3067, 3024, 2995, 2849, 1589, 1555, 1464, 1443. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.17 (dd, 1H, *J*= 5.76, 17.44 Hz, pyrazoline proton), 3.86 (dd, 1H, *J* = 12.34, 17.46 Hz, pyrazoline proton), 5.98 (dd, 1H, *J* = 5.74, 12.26 Hz, pyrazoline proton), 7.30-7.40 (m, 7H, arom. protons), 7.49 (d, 2H, *J* = 8.20 Hz, arom. protons), 7.63 (d, 2H, *J* = 8.52 Hz, arom. protons), 7.68 (d, 1H, *J* = 9.36 Hz, pyridazine proton), 7.77 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.89 (d, 2H, *J* = 7.12 Hz, arom. protons). Anal.Calcd. for $C_{26}H_{18}ClF_3N_4$ (478.90): C, 65.21; H, 3.79; N, 11.70. Found: C, 65.34; H, 3.76; N, 11.82.

4.1.1.12. 3-[3-(4-Chlorophenyl)-5-(4-methoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenyl pyridazine **8l**

Obtained from the reaction of **3l** with **7**, mp 173-175˚C, yield 70% (0.46 g). IR: ν_{max}/ cm^{-1} 3055, 3038, 2984, 2957, 2918, 2837, 1589, 1574, 1549, 1464, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.18 (dd, 1H, *J* = 5.14, 17.42 Hz, pyrazoline proton), 3.66 (s, 3H, OCH_3), 3.77 (dd, 1H, *J* = 12.14, 17.42 Hz, pyrazoline proton), 5.93 (dd, 1H, *J* = 4.94, 11.98 Hz, pyrazoline proton), 6.74 (d, 2H, *J* = 8.60 Hz, arom. protons), 7.21 (d, 2H, *J* = 8.60 Hz, arom. protons), 7.30-7.38 (m, 5H, arom. protons), 7.63 (d, 3H, *J* = 8.80 Hz, arom. protons + pyridazine proton), 7.71 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.88 (d, 2H, *J* = 7.44 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 42.7 (pyrazoline CH_2), 55.3 (OCH_3), 61.6 (pyrazoline CH), 114.2, 115.2, 125.3, 126.1, 127.4, 128.7, 128.8, 129.0, 130.6, 134.3, 135.4, 136.8, 150.3, 152.6, 155.5, 158.9 (aromatic carbons). Anal.Calcd. for $C_{26}H_{21}ClN_4O$ (440.92): C, 70.82; H, 4.80; N, 12.71. Found: C, 70.94; H, 4.85; N, 12.83.

4.1.1.13. 3-[3-(4-Chlorophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8m**

Obtained from the reaction of **3m** with **7**, mp 195-197˚C, yield 57% (0.40 g). IR: ν_{max}/ cm^{-1} 3057, 3003, 2951, 2934, 2832, 1589, 1551, 1508, 1462, 1437. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.05 (dd, 1H, *J* = 4.96, 17.32 Hz, pyrazoline proton), 3.66 (s, 3H, OCH_3), 3.70-3.77 (m, 4H, OCH_3 + pyrazoline proton), 6.10 (dd, 1H, *J* = 4.92, 11.96 Hz, pyrazoline proton), 6.25 (d, 1H, *J* = 8.36 Hz, arom. protons), 6.40 (s, 1H, arom. proton), 6.89 (d, 1H, *J* = 8.40 Hz, arom. proton), 7.29-7.38 (m, 5H, arom. protons), 7.62 (d, 2H, *J* = 8.32 Hz,

arom. protons), 7.65 (d, 1H, *J* = 9.48 Hz, pyridazine proton), 7.74 (d, 1H, *J* = 9.36 Hz, pyridazine proton), 7.91 (d, 2H, *J* = 7.60 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 41.7 (pyrazoline CH_2), 55.4 (OCH_3), 55.5 (OCH_3), 57.8 (pyrazoline CH), 99.1, 103.8, 114.9, 121.8, 125.2, 126.1, 126.9, 127.4, 128.7, 128.9, 130.9, 135.2, 136.8, 151.4, 152.2, 155.7, 157.4, 160.2 (aromatic carbons). Anal.Calcd. for $C_{27}H_{23}ClN_4O_2$ (470.95): C, 68.86; H, 4.92; N, 11.90. Found: C, 69.03; H, 4.98; N, 12.04.

4.1.1.14. 3-[3-(4-Chlorophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8n**

Obtained from the reaction of **3n** with **7**, mp 196-198˚C, yield 50% (0.38 g). IR: ν_{max}/ cm^{-1} 3032, 2967, 2916, 2887, 1587, 1576, 1547, 1458, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.17 (dd, 1H, *J* = 5.50, 17.42 Hz, pyrazoline proton), 3.71 (s, 3H, OCH_3), 3.72 (s, 6H, $2OCH_3$), 3.79 (dd, 1H, *J* = 12.24, 17.36 Hz, pyrazoline proton), 5.87 (dd, 1H, *J* = 5.48, 12.12 Hz, pyrazoline proton), 6.45 (s, 2H, arom. protons), 7.32-7.40 (m, 5H, arom. protons), 7.63 (d, 2H, *J* = 8.40 Hz, arom. protons), 7.68 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.76 (d, 1H, *J* = 9.36 Hz, pyridazine proton), 7.91 (d, 2H, *J* = 7.48 Hz, arom. protons). Anal.Calcd. for $C_{28}H_{25}ClN_4O_3$ (500.98): C, 67.13; H, 5.03; N, 11.18. Found: C, 67.24; H, 5.08; N, 11.27.

4.1.1.15. 3-[3-(4-methoxyphenyl)-5-phenyl-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8o**

Obtained from the reaction of **3o** with **7**, mp 201-202˚C, yield 48% (0.29 g). IR: ν_{max}/cm^{-1} 3082, 3024, 2916, 2849, 1609, 1591, 1547, 1516, 1456, 1437. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.30 (dd, 1H, *J* = 5.22, 17.30 Hz, pyrazoline proton), 3.87-3.95 (m, 4H, OCH_3 + pyrazoline proton), 6.02 (dd, 1H, *J* = 5.22, 12.06 Hz, pyrazoline proton), 6.98 (d, 2H, *J* = 8.84 Hz, arom. protons), 7.21-7.33 (m, 5H, arom. protons), 7.37-7.47 (m, 5H, arom. protons), 7.73 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.74 (d, 2H, *J* = 8.76 Hz, arom. protons), 7.83 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.99 (d, 2H, *J* = 7.20 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 43.1 (pyrazoline CH_2), 55.4 (OCH_3), 61.8 (pyrazoline CH), 114.2, 115.0, 124.7, 125.2, 126.1, 127.3, 127.8, 128.6, 128.7, 128.8,

136.9, 142.5, 151.4, 152.1, 155.7, 160.8 (aromatic carbons).Anal.Calcd. for $C_{26}H_{22}N_4O$ (406.49): C, 76.83; H, 5.46; N, 13.78. Found: C, 76.97; H, 5.52; N, 13.89.

4.1.1.16. 3-[5-(4-Chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8p**

Obtained from the reaction of **3p** with **7**, mp 203-205˚C, yield 73% (0.48 g). IR: ν_{max}/ cm^{-1} 3046, 3026, 2953, 2928, 2835, 1609, 1593, 1551, 1516, 1468, 1441. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.26 (dd, 1H, *J* = 5.42, 17.34 Hz, pyrazoline proton), 3.87-3.94 (m, 4H, OCH_3 + pyrazoline proton), 5.96 (dd, 1H, *J* = 5.40, 12.08 Hz, pyrazoline proton), 6.98 (d, 2H, *J* = 8.84 Hz, arom. protons), 7.27-7.34 (m, 4H, arom. protons), 7.40-7.48 (m, 3H, arom. protons), 7.73-7.75 (m, 3H, 2H arom. protons + pyridazine proton), 7.82 (d, 1H, J =9.40 Hz, pyridazine proton), 7.98 (d, 2H, *J* = 7.08 Hz, arom. protons). Anal.Calcd. for $C_{26}H_{21}ClN_4O$ (440.94): C, 70.82; H, 4.80; N, 12.71. Found: C, 70.95; H, 4.84; N, 12.86.

4.1.1.17. 3-[5-(4-Fluorophenyl)-3-(4-methoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenyl pyridazine **8q**

Obtained from the reaction of **3q** with **7**, mp 168-170˚C, yield 52% (0.33 g). IR: ν_{max}/ cm^{-1} 3038, 2959, 2920, 2837, 1609, 1593, 1549, 1506, 1468, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.26 (dd, 1H, *J* = 5.30, 17.34 Hz, pyrazoline proton), 3.86- 3.93 (m, 4H, OCH_3 + pyrazoline proton), 5.98 (dd, 1H, *J* = 5.28, 12.04 Hz, pyrazoline proton), 6.97-7.02 (m, 4H, arom. protons), 7.34-7.48 (m, 5H, arom. protons), 7.71- 7.75 (m, 3H, 2H arom. protons + pyridazine proton), 7.82 (d, 1H, J= 9.40 Hz, pyridazine proton), 7.99 (d, 2H, *J* = 7.08 Hz, arom. protons). Anal.Calcd. for $C_{26}H_{21}FN_4O$ (424.48): C, 73.57; H, 4.99; N, 13.20. Found: C, 73.73; H, 5.03; N, 13.37.

4.1.1.18. 3-[3-(4-Methoxyphenyl)-5-(4-(trifluoromethyl)phenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8r**

Obtained from the reaction of **3r** with **7**, mp 220-222˚C, yield 40% (0.28 g). IR: ν_{max}/ cm^{-1} 3060, 2928, 2839, 1593, 1514, 1464, 1441. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.17 (dd, 1H, *J* = 5.60, 17.36 Hz, pyrazoline proton), 3.79 (s, 3H, OCH_3), 3.85 (dd, 1H, *J* = 12.20, 17.36 Hz, pyrazoline proton), 5.94 (dd, 1H, J = 5.60, 12.16 Hz, pyrazoline proton), 6.89

(d, 2H, *J* = 8.80 Hz, arom. protons), 7.29-7.41 (m, 5H, arom. protons), 7.48 (d, 2H, *J* = 8.24 Hz, arom. protons), 7.63-7.67 (m, 3H, 2 arom. protons+ pyridazine proton), 7.75 (d, 1H, J = 9.40 Hz, pyridazine proton), 7.89 (d, 2H, J = 7.20 Hz, arom. protons). Anal.Calcd. for $C_{27}H_{21}F_3N_4O$ (474.49): C, 68.35; H, 4.46; N, 11.81. Found: C, 68.44; H, 4.52; N, 11.96.

4.1.1.19. 3-[3,5-bis(4-Methoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8s**
Obtained from the reaction of **3s** with **7**, mp 195-196˚C, yield 64% (0.42 g). IR: ν_{max}/cm^{-1} 3042, 3009, 2949, 2934, 2832, 1595, 1549, 1514, 1466, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.29 (dd, 1H, *J* = 5.08, 17.32 Hz, pyrazoline proton), 3.77 (s, 3H, OCH_3), 3.84-3.91 (m, 4H, OCH_3 + pyrazoline proton), 5.97 (dd, 1H, *J* = 5.06, 11.98 Hz, pyrazoline proton), 6.84 (d, 2H, J= 8.64 Hz, arom. protons), 6.98 (d, 2H, *J* = 8.80 Hz, arom. protons), 7.32 (d, 2H, J = 8.68 Hz, arom. protons), 7.37-7.48 (m, 3H, arom. protons), 7.71 (d, 1H, *J* = 9.44 Hz, pyridazine proton), 7.75 (d, 2H, *J* = 8.80 Hz, arom. protons), 7.80 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 7.99 (d, 2H, *J* = 7.24 Hz, arom. protons). Anal.Calcd. for $C_{27}H_{24}N_4O_2$ (436.52): C, 74.29; H, 5.54; N, 12.83. Found: C, 74.53; H, 5.59; N, 13.01.

4.1.1.20. 3-[5-(2,4-Dimethoxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8t**
Obtained from the reaction of **3t** with **7**, mp 183-185˚C, yield 59% (0.41 g). IR: ν_{max}/cm^{-1} 3051, 2916, 2833, 1607, 1589, 1551, 1508, 1464, 1437. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.15 (dd, 1H, *J* = 4.94, 17.26 Hz, pyrazoline proton), 3.76 (s, 3H, OCH_3), 3.81-3.88 (m, 7H, 2 OCH_3 + pyrazoline proton), 6.17 (dd, 1H, *J* = 4.92, 11.84 Hz, pyrazoline proton), 6.34 (dd, 1H, *J* = 2.14, 8.38 Hz, arom. protons), 6.50 (d, 1H, *J* = 2.12 Hz, arom. protons), 6.94-7.00 (m, 3H, arom. protons), 7.37-7.48 (m, 3H, arom. protons + pyridazine proton), 7.73 (d, 2H, *J* = 8.72 Hz, arom. protons), 7.83 (d, 1H, *J* = 9.40 Hz, pyridazine proton), 8.01 (d, 2H, *J* = 7.40 Hz, arom. Protons). Anal.Calcd. for $C_{28}H_{26}N_4O_3$ (466.54): C, 72.09; H, 5.62; N, 12.01. Found: C, 72.24; H, 5.64; N, 12.13.

4.1.1.21. 3-[3-(4-Methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydropyrazol-1-yl]-6-phenylpyridazine **8u**

Obtained from the reaction of **3u** with **7**, mp 193-195˚C, yield 41% (0.31 g). IR: ν_{max}/ cm^{-1} 3050, 2980, 2963, 2837, 1607, 1591, 1508, 1462, 1439. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.18 (dd, 1H, J = 5.52, 17.32 Hz, pyrazoline proton), 3.71 (s, 3H, OCH_3), 3.72 (s, 6H, 2 OCH_3), 3.75-3.83 (m, 4H, OCH_3 + pyrazoline proton), 5.83 (dd, 1H, J = 5.48, 12.04 Hz, pyrazoline proton), 6.47 (s, 2H, arom. protons), 6.88 (d, 2H, J = 8.84 Hz, arom. protons), 7.29-7.39 (m, 3H, arom. protons), 7.65 (d, 2H, J = 8.76 Hz, arom. protons), 7.66 (d, 1H, J = 9.36 Hz, pyridazine proton), 7.76 (d, 1H, J = 9.40 Hz, pyridazine proton), 7.92 (d, 2H, J = 7.24 Hz, arom. protons). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 43.4 (pyrazoline CH_2), 55.4 (OCH_3), 56.1 ($2OCH_3$), 60.7 (OCH_3), 62.1 (pyrazoline CH), 102.5, 114.2, 115.2, 124.6, 125.2, 126.1, 127.8, 128.8, 136.8, 137.1, 138.4, 151.6, 152.3, 153.6, 155.9, 160.9 (aromatic carbons). Anal.Calcd. for $C_{29}H_{28}N_4O_4$ (496.56): C, 70.15; H, 5.68; N, 11.28. Found: C, 70.29; H, 5.72; N, 11.44.

4.2. In-vitro MTT assay

Human cancer cell lines of different origins purchased from ATCC were used for evaluation of the anticancer effect of the target pyrazolines. MCF7 (ATCC; HTB-22), CaCo-2 (ATCC; HTB-37), HepG2 (ATCC; HB-8065) & A549 (ATCC; CRM-CCL-185). Cells were cultured and maintained as monolayer in either Dulbecco's modified Eagle's medium (DMEM) for A549 and HepG2, or Rose Well Park Memorial Institute medium (RPMI) for MCF-7 and CaCo-2 cell lines. Each medium was supplemented with 10% of fetal calf serum (Lonza), antibiotics: 100 IU/mL of penicillin (Lonza) and 100 mg/mL of streptomycin (Lonza). All cells were cultured in 10 cm diameter culture plates at 37^0C in 100% humidity atmosphere and 5% of CO_2 [56]. Only viable cells were used in the assay. Stock solutions of 1M from the tested compounds were prepared in serum free culture medium containing 1% (v/v) dimethylsulfoxide (DMSO). Cells were seeded overnight in 96 multiwells plate (10^3 cells/well) before treatment with the prepared compounds to allow the attachment of cells to the wall of the plate. A further dilution of the stock solution from each compound in complete medium (10% serum) was added to the seeded cells at concentrations of 0, 5, 10, 50, and 100 µM. Each cell line was

incubated with the compounds for 24 h at standard culture conditions. The potential cytotoxicity of the tested compounds was evaluated at 24 h post incubation with the respective compounds using MTT colorimetric assay (WST-1 premix cell proliferation assay – Takara, Germany), measured at a wavelength of 450 nm. Compounds mediated cellular toxicity was calculated using the following formula (specified by the kit protocol); % Cell viability = [(Absorbance of treated cells – absorbance of blank) / (Absorbance of control cells – Absorbance of blank)] x 100.

After 24 h incubation with the cells, control (untreated cells) did not exhibit significant changes compared to the 1% DMSO vehicle. The IC_{50}, mean value and standard error were determined, and data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was done using one-way ANOVA Tukey's Multiple Comparison Test via GraphPad Prism 5.0 software.

4.3. Evaluation of Cell Death Pathways

Flow cytometry was applied in order to determine the mechanism of cancer cell death for each cell line. Fluorescent markers of fluorescein isothiocyanate (FITC)-Annexin V and Ethidium Homodimer III (EthD-III) from (Biotium, USA) for detection of apoptotic and necrotic cell deaths were used respectively. A number of 2-3 x 10^5 cells/ml treated with the LC_{50} – PDT conditions were re-suspended in 1 ml of 1X binding buffer diluted in distilled water, then 5 µl of FITC-Annexin V, together with 5 µl of EthD-III were added to 100 µl of cell suspension and incubated at room temperature for 15 min in dark. 400 µl of 1X binding buffer was added to the cell suspension and fluorescence was measured within 1 h of staining by fluorescence associated cell sorter (FACS) analysis.

4.4. Caspase-3 activation assay

The activity of caspase-3 was determined at Vacsera, Dokki, Cairo, Egypt, using MaxDiscovery™caspase-3 colorimetric detection kit, Bioo Scientific Corporation (BIOO), USA. Briefly, MCF-7 cells (5×10^5 cells/well) in 6-well plate were treated with IC_{50} concentrations of compound **8k** for 24 h. The cell culture medium was gently removed from the control and treated culture wells and then the cells were lysed by

adding cell lysis buffer (1000 µL) to each culture well. The plate was gently shaken for 10 minutes to facilitate cell lysis and sample homogenization. Caspase-3 Substrate (100 µL) was diluted into 10 mL of Reaction Buffer. In the reaction microplate, 100 µL Caspase-3 Reaction Buffer was added to each well, followed by 100 µL of cell lysate. The absorbance was measured using a plate reader for the increase in absorbance at 405 nm in 30 minutes. Caspase-3 activity was expressed as the change of the activity in treated cancer cells compared to the untreated controls [57].

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Highlights:

- Pyrazoline derivatives **8a-u** were synthesized under basic condition.
- All compounds were screened for their cytotoxic activity against four cell lines.
- Their cytotoxicity is mainly due to apoptotic rather than necrotic induction.
- Compound **8k** revealed promising activity against HepG-2, MCF-7 and CaCo-2.
- Compound **8k** resulted in 5 fold increase in caspase-3 activity relative to control.