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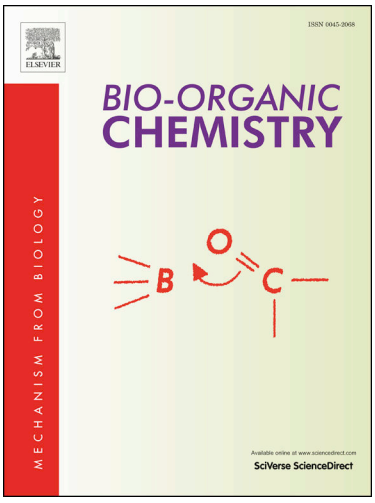

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PII: S0045-2068(19)32114-5
DOI: https://doi.org/10.1016/j.bioorg.2020.103780
Reference: YBIOO 103780

To appear in: *Bioorganic Chemistry*

Received Date: 9 December 2019
Revised Date: 17 March 2020
Accepted Date: 19 March 2020

Please cite this article as: R.F. George, M. Kandeel, D.Y. El-Ansary, A.M. ElKerdawy, Some 1,3,5-Trisubstituted Pyrazoline Derivatives Targeting Breast Cancer: Design, Synthesis, Cytotoxic activity, EGFR inhibition and Molecular docking, *Bioorganic Chemistry* (2020), doi: https://doi.org/10.1016/j.bioorg.2020.103780

Some 1,3,5-Trisubstituted Pyrazoline Derivatives Targeting Breast Cancer: Design, Synthesis, Cytotoxic activity, EGFR inhibition and Molecular docking

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Declarations of interest: none

Abstract

Different 1,3,5-trisubstituted pyrazoline derivatives **2a-c**, **3-c**, **4a-f**, **6a-c**, **7a-f** and **8a-d** were prepared via condensation reaction of the appropriate chalcone **1a-c** or **5a-c** with various hydrazine derivatives. All compounds were screened for their cytotoxicity against breast MCF-7 cancer cell line and the normal fibroblasts WI-38. Thirteen compounds **2a**, **3a**, **3c**, **4a-d**, **6c**, **7d**, **7e**, **8b**, **8d** and **8f** revealed promising cytotoxicity against MCF-7 compared to the reference standard staurosporine and they were safe to the normal fibroblasts WI-38. In addition, compounds **3c**, **6c**, **7d**, **8b** and **8d** elicited higher cytotoxicity than erlotinib and exhibited promising EGFR inhibitory activity at submicromolar level comparable to that of erlotinib except for compound **8b** that may exert its cytotoxicity via another mechanism besides EGFR inhibition. Molecular docking of **3c**, **6c**, **7d**, **8b** and **8d** in the active site of EGFR confirmed the obtained results.

Key words: Pyrazolines; Chalcones; Hydrazine; MCF7; EGFR

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1. Introduction

Breast cancer is one of the most common cancer types in both developed and developing countries. Despite the presence of advanced diagnostic techniques for its early detection, breast cancer occupies the first place in the detected new cancer cases in the US annually (268,600 cases), in addition to its high death rate of 41,760 deaths annually [1]. These records reflect the presence of a defect in the breast cancer treatment which may result from the emergence of a resistance to the used therapies [1]. Therefore, there is an urgent need for the discovery of more efficient anticancer agents acting on clinically validated targets.

Epidermal Growth Factor Receptor (EGFR) is one of the tyrosine kinases (TKs) that play a crucial role in the regulation of several cellular functions such as cell growth, survival, proliferation, differentiation, and apoptosis [2]. However, in many cancer types as hepatocellular carcinoma, breast cancer, non-small cell lung cancer, pancreatic cancer, colorectal carcinoma, melanoma, and glioblastoma, there are significant resistance to the used EGFR inhibitors [3]. Therefore, four generations of EGFR inhibitors have been discovered to combat different types of cancer and to solve the problem of resistance emerged from each precedent generation [4].

Pyrazoline is a privileged scaffold found in many bioactive agents with diverse biological activities [5-10]. For example, compound **I** and the thiazolyl-pyrazoline derivative **II** displayed potent cytotoxic activity against breast cancer cell line (MCF-7) with IC_{50} of 0.08 and 0.07 μM, respectively, through EGFR inhibition with IC_{50} of 0.07 and 0.06 μM, respectively, whereas, the positive control erlotinib, in these studies, showed IC_{50} of 0.03 and 0.02 μM, respectively [10,11]. Moreover, the pyrazoline derivative **III** exhibited promising cytotoxicity against MCF-7 with IC_{50} of 0.16 μM in comparison to sorafenib (IC_{50} = 0.19 μM) which used as reference standard in this study [12]. Additionally, the 2,4-disubstituted thiazole-based pyrazoline derivative **IV** elicited equipotent cytotoxic activity against breast cancer cell line (MCF-7) compared to cisplatin with IC_{50} of 7.50 μM [13] (Figure 1).

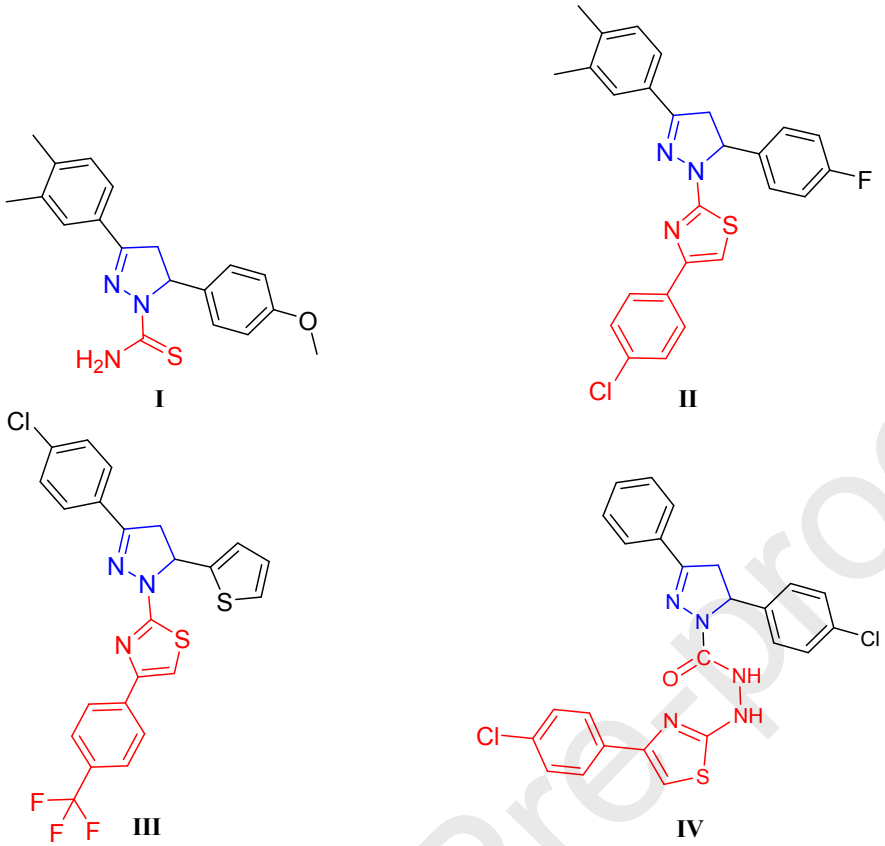


Figure 1. Reported 1,3,5-trisubstituted-4,5-dihydro-1*H*-pyrazolines with cytotoxic activity against breast cancer cell line (MCF-7) and/or EGFR inhibitory activity.

Inspired by these data, this work focuses on the synthesis of 1,3,5-trisubstituted-4,5-dihydro-1*H*-pyrazolines with styryl and aryl moieties incorporated at positions 3 and/or 5 (General formulae A and B, Figure 2) in order to study the effect of this positional isomerism on the cytotoxic activity. Furthermore, different substituents at the *para* position of the aryl and styryl moieties will be used to investigate the impact of their electronic effects on the obtained activity. Additionally, at the pyrazoline position 1, various groups will be inserted as acetyl (compounds **2a-c**, **6a-c**), carboxamide (compounds **3a-c**and **7a-c)** and its bioisostere carbothioamide (compounds **7d-f**) to study the effect of these structural modifications on the obtained activity. Moreover, attachment of phenyl or *p*-methoxyphenyl moiety at position 1 of the pyrazoline ring was carried out (compounds **4a-c**, **8a-f**) to study the influence of their lipophilicity and steric effect on the obtained activity (Figure 2).

All the designed compounds will be screened for their cytotoxicity against breast cancer cell line (MCF-7) as well as normal fibroblasts (WI-38) to ensure their safety to normal cells and selectivity towards cancerous cells. Additionally, compounds eliciting promising anticancer activity will be tested for their EGFR inhibitory activity to shed light on their possible mode of action. Furthermore, molecular docking simulation of the most active EGFR inhibitors in the receptor active site will be performed to validate the obtained experimental results and to illustrate their binding pattern and binding interactions with the key amino acids in the EGFR active site.

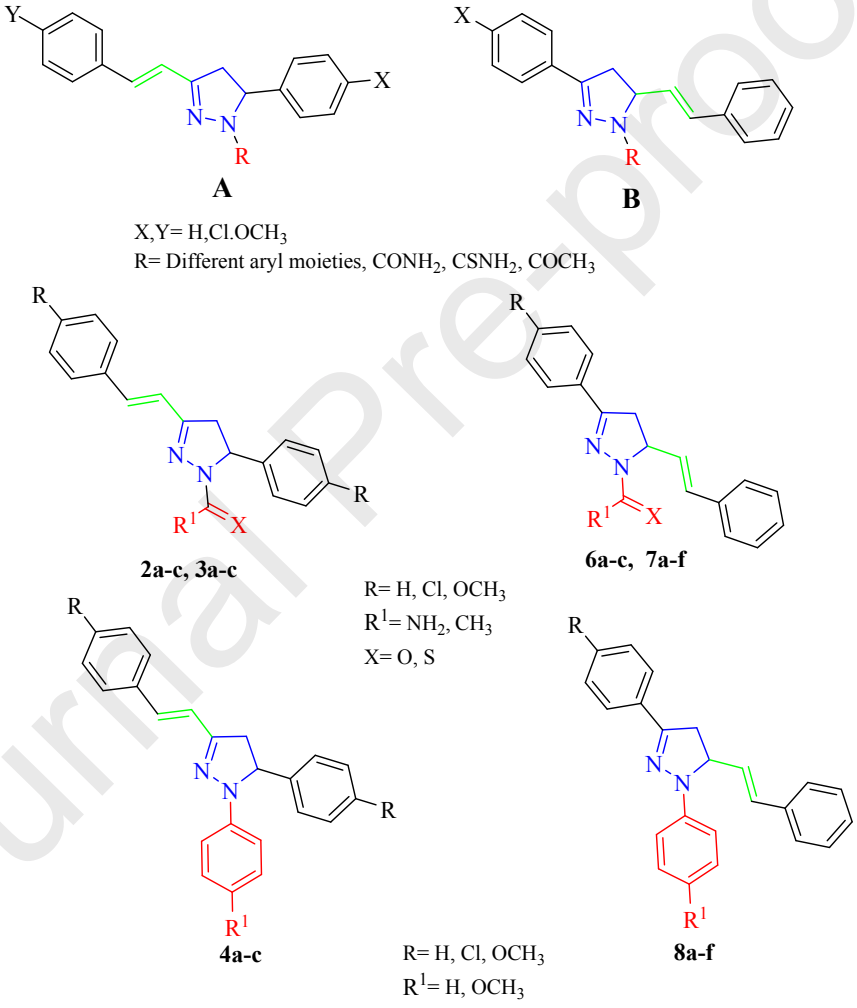


Figure 2. Structure of the target 1,3,5-trisubstituted-4,5-dihydro-1*H*-pyrazolines

2. Results and discussion

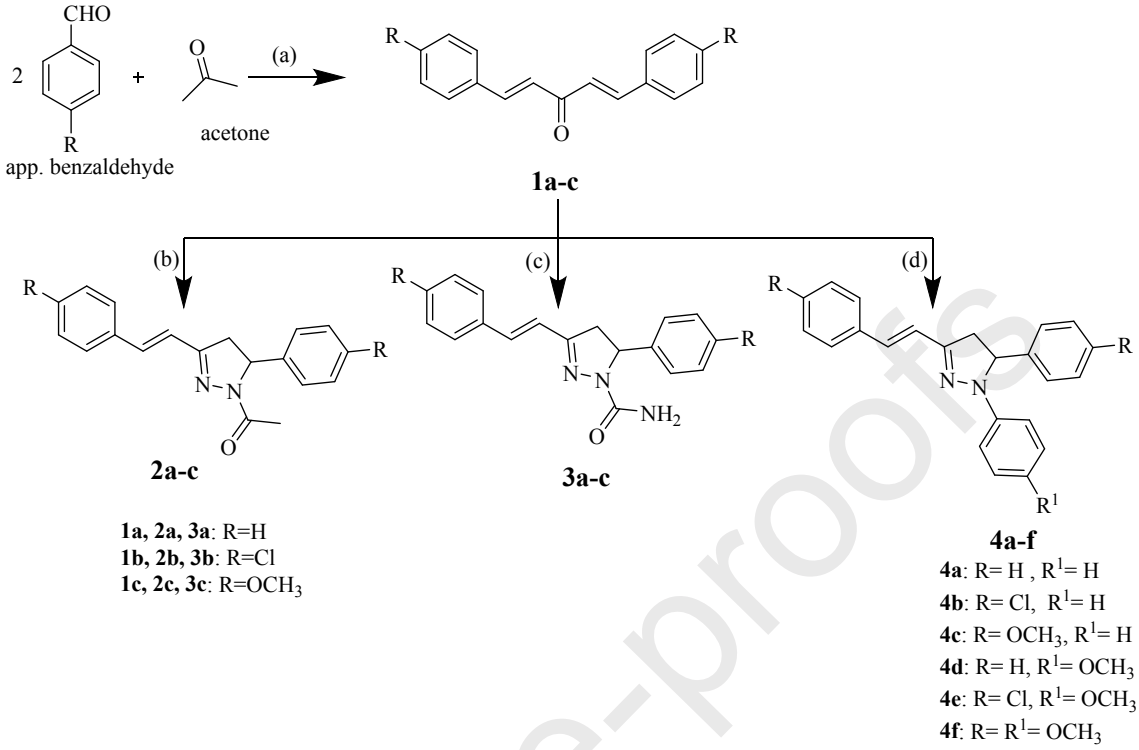
2.1. Chemistry

The synthetic pathways used for preparation of the target compounds are illustrated in Schemes 1 and 2. (1*E*, 4*E*)-1,5-diarylpenta-1,4-dien-3-ones (**1a-c**) were prepared as reported via Claisen Schmidt reaction of the appropriate benzaldehyde and acetone in ethanol under basic conditions [14-16]. Similarly, the condensation of cinnamaldehyde and the appropriate acetophenones in ethanol in the presence of sodium hydroxide afforded the chalcones **5a-c** [17, 18]. Furthermore, the reaction of **1a-c** or **5a-c** with hydrazine hydrate in glacial acetic acid afforded the reported 1-acetyl-5-aryl-3-styryl-4,5-dihydro-1*H*-pyrazolines (**2a-c)** or their positional isomers **6a-c**, respectively [19,20]. ^{1}H NMR spectra of **2a-c** confirmed the cyclization due to the appearance of 3 doublet of doublets at 2.89-3.05, 3.51-3.62 and 5.42-5.57 ppm, respectively assigned for H_A, H_M and H_X of the pyrazoline ring in addition to a singlet signal at 2.27-2.40 ppm attributed to the acetyl methyl protons. Furthermore, two doublets for the two olefinic protons appeared at 6.63-6.79 and 6.99-7.14 ppm, respectively with high coupling constant (J = 16.32-16.40 Hz) confirming *E* conformation of olefinic protons at the double bond. On the other hand, ^{1}H NMR spectrum of **6a-c** presented a singlet signal at δ 2.43-2.45 ppm corresponding to CH_3 protons of the acetyl group in addition to two doublets of doublet for H_A, H_M protons of pyrazoline ring at 3.11-3.16, 3.54-3.58, respectively. Furthermore, a multiplet for H_X of pyrazoline ring appeared at 5.29-5.35 ppm due to coupling with H_A, H_M at C-4 of pyrazoline as well as olefinic proton at side chain. Consequently, this olefinic proton appeared as doublet of doublet at 6.22-6.23 ppm due to coupling with H_x of pyrazoline and the neighboring olefinic proton that appeared as a doublet at 6.61-6.63 ppm.

Moreover, reaction of **1a-c** with semicarbazide hydrochloride in ethanol in the presence of sodium hydroxide resulted in formation of the reported 1-carboxamide pyrazoline derivative **3a** [21] and the new congeners **3b** and **3c**. The reaction of **5a-c** with either semicarbazide hydrochloride or thiosemicarbazide adopting the same reaction conditions resulted in compounds **7a-f**. IR spectrum of compounds **3a-c** showed NH_2 bands at 3478-3441, 3498-3398 cm^{-1} and C=O band at 1676-1660 cm^{-1}. ^{13}C NMR of **3b** and **3c** revealed signals for CH_2 and CH carbons of the pyrazoline ring at 42.0 and 59.4-59.5 ppm,

respectively along with C=O signal at 155.0-159.0 ppm, in addition to two signals at 55.3 and 55.4 ppm due to two OCH_3 groups of compound **3c**. IR spectrum of **7a-f** showed NH_2 bands at 3495-3275, 3402-3197 cm^{-1}, C=O band at 1681-1674 cm^{-1} for compounds **7a-c** and C=S band at 1253-1246 cm^{-1} for compound **7d-f**. ^1H NMR of compounds **7a-f** showed, two doublets of doublet for H_A, H_M at 3.03-3.16, 3.49-3.61, respectively and a multiplet for H_X of pyrazoline ring at 5.10-5.26 ppm along with a doublet of doublet and a doublet at 6.18-6.29 and 6.54-6.64 ppm, respectively attributed to the olefinic protons. Moreover, an exchangeable singlet signal of NH_2 protons appeared at 5.30-5.52 ppm which disappeared upon deuteration.

Additionally, 1-phenyl-4,5-dihydro-1*H*-pyrazoline derivatives **4a-c** or their analogs **8a-c** were obtained according to the reported procedure [14, 20, 22] via reaction of phenylhydrazine with the chalcones **1a-c** or **5a-c**, respectively, in absolute ethanol in the presence of glacial acetic acid. However, the reaction of *p*-methoxyphenylhydrazine hydrochloride with either **1a-c** or **5a** was carried out in ethanol to give the corresponding 1-(4-methoxyphenyl)-pyrazoline analogs **4d-f** or **8d**, respectively. Whereas, using the same conditions in case of reaction of **5b** and **5c** with *p*-methoxyphenylhydrazine hydrochloride affording the open condensation products **8e** and **8f** instead of their pyrazoline congeners. ^1H NMR spectrum of **4a-f** confirmed the pyrazoline ring formation by the presence of three doublets of doublet for H_A, H_M and H_X of the pyrazoline ring at 2.94-3.03, 3.65-3.86 and 5.18-5.26 ppm, respectively, and two doublets for two olefinic proton at 6.46-6.54 ppm and 7.07-7.38 ppm. ^{13}C NMR of **4d-f** showed a signal referred to CH_2 and CH carbons of the pyrazoline ring at 42.2-45.4 and 61.0-64.8 ppm, respectively along with the methoxy signal at 55.3-55.6 ppm. ^1H NMR spectra of **8a-d** exhibited two doublets of doublet for H_A, H_M at 3.12-3.16, 3.63-3.67, respectively and a multiplet for H_X of pyrazoline ring at 4.80-5.16 ppm, in addition to a doublet of doublet and a doublet at 6.15-6.36 and 6.58-6.92 ppm attributed to the olefinic protons. On the other hand, ^1H NMR spectra of **8e** and **8f** lacked the AMX pattern of pyrazoline protons and showed a singlet signal at δ 5.32-6.95 ppm corresponding to NH proton and two doublets for four olefinic protons at 6.75-6.85 ppm and 7.04-7.16 ppm negating the pyrazoline ring formation and indicating that only the condensed products **8e** and **8f** were obtained.

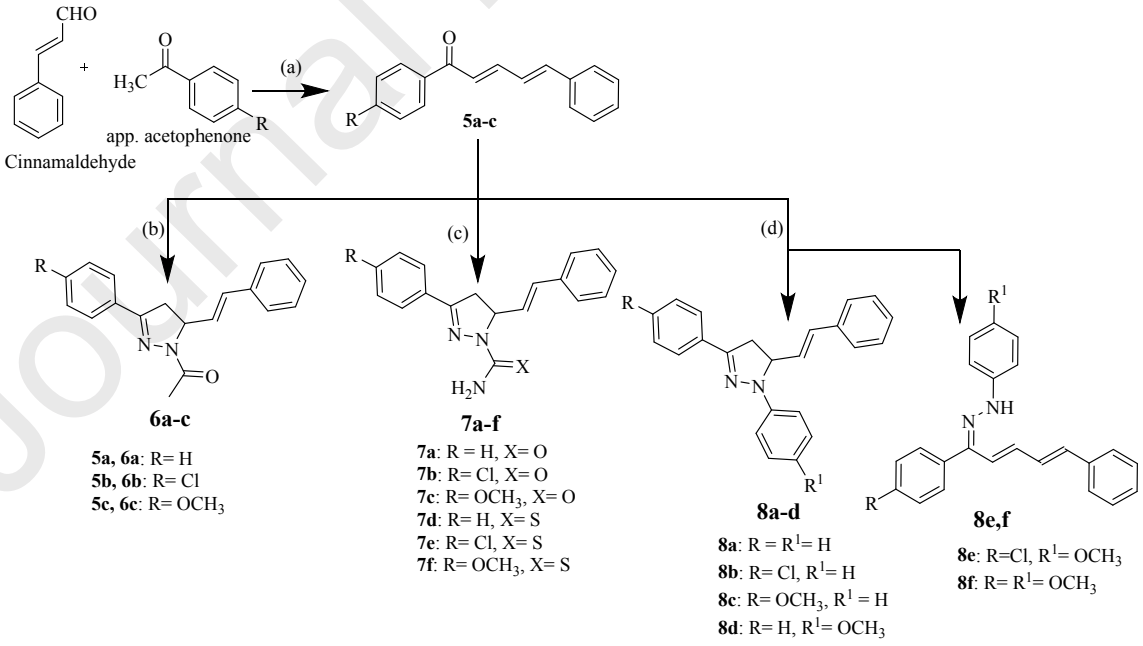


1a, 2a, 3a: R=H
1b, 2b, 3b: R=Cl
1c, 2c, 3c: R=OCH_3

4a: R= H , R^1= H
4b: R= Cl, R^1= H
4c: R= OCH_3, R^1= H
4d: R= H, R^1= OCH_3
4e: R= Cl, R^1= OCH_3
4f: R= R^1= OCH_3

Reagents and conditions: (a) NaOH, absolute ethanol, RT, 1.5h; (b) Hydrazin hydrate**,** glacial acetic acid, 8h; (c) Semicarbazide.HCl, NaOH, EtOH, 8h; (d)Phenyl hydrazine , glacial acetic acid, EtOH, 8h or *p*-methoxy phenyl hydrazine HCl, EtOH, 8h.

Scheme 1. The synthetic pathway for compounds **2a-c**, **3a-c** and **4a-f**



5a, 6a: R= H
5b, 6b: R= Cl
5c, 6c: R= OCH_3

7a: R = H, X= O
7b: R= Cl, X= O
7c: R= OCH_3, X= O
7d: R= H, X= S
7e: R= Cl, X= S
7f: R= OCH_3, X= S

8a: R = R^1= H
8b: R= Cl, R^1= H
8c: R= OCH_3, R^1 = H
8d: R= H, R^1= OCH_3

8e: R=Cl, R^1= OCH_3
8f: R= R^1= OCH_3

Reagents and conditions: (a) NaOH, EtOH, RT, 20h for 5a and 1h for 5b,c; (b) Hydrazine hydrate**,** glacial acetic acid 8h; (c) Semicarbazide HCl or thiosemicarbazide, NaOH, EtOH, 8h; (d) Phenyl hydrazine, glacial acetic acid, EtOH, 8h or *p*-methoxy phenyl hydrazine HCl, EtOH, 8h.

Scheme 2. Synthetic pathways for compounds **6a-c, 7a-f** and **8a-f.**

2.2. *In vitro cytotoxic activity*

It should be noted that although some of the targeted final compounds in the present investigation such as **2a-c**, **3a**, **4a-c**, **6a-c**, **8a** and **8c** are previously reported, none of them has been screened for potential cytotoxic activity. Therefore, they were synthesized and screened for their cytotoxicity to clarify the SAR study. Thus, twenty-seven synthesized compounds; **2a-c**, **3a-c**, **4a-f**, **6a-c**, **7a-f** and **8a-f** were evaluated for their *in vitro* cytotoxicity against breast cancer cell line (MCF-7) in addition to normal fibroblast cells (WI38) using MTT assay [23-25]. Staurosporine and erlotinib were used as reference standards; staurosporine is a cell permeable indolocarbazole alkaloid isolated from *Streptomyces staurosporeus* exhibiting an anticancer activity through a potent, non-selective inhibition of protein kinases [26]. From the obtained results (Table 1), it can be observed that the tested compounds revealed moderate to potent cytotoxicity against breast cancer cell line (MCF-7) with IC_{50} ranged between 3.79 – 37.73 μM compared to staurosporine and erlotinib (which showed IC_{50} = 10.61 and 4.74 μM, respectively). Compounds **2a**, **3a**, **3c**, **4a-d**, **6c**, **7d**, **7e**, **8b**, **8d** and **8f** exhibited higher activity compared to staurosporine, while compounds **3c**, **6c**, **7d**, **8b** and **8d** elicited comparable activity to erlotinib.

Furthermore, the following structure activity relationships could be concluded:

In the 1-acetylpyrazoline derivatives **2a-c** and their positional isomers **6a-c**, it was found that the presence of styryl moiety at position 3 and phenyl ring at position 5 of the pyrazoline ring **2a** represented the highest activity (IC_{50} = 6.62 μM) followed by the 4-methoxyphenyl derivative **2c** and its 4-chloro analog **2b** (IC_{50} = 17.47 and 29.19 μM, respectively). On the other hand, changing the position of styryl moiety to position 5 of the pyrazoline ring resulted in a decrease in the activity in case of the unsubstituted **6a** and 4-chloro derivatives **6b** (IC_{50} = 22.01 and 31.61 μM, respectively) in comparison to their analogs **2a** and **2b**. Whereas, the 4-methoxyphenyl derivative **6c** revealed the highest activity among these series with IC_{50} of 4.33 μM which was superior to both sataurosporine and erlotinib.

Furthermore, the1-carboxamide pyrazoline derivative with 4-methoxyphenyl substituent at position 5, compound **3c,** displayed a significant activity (IC_{50} = 4.33 μM) followed by

the unsubstituted **3a** and 4-chloro **3b** congeners (IC_{50} = 10.59 and 16.87 μM, respectively). The 5-styryl-1-carboxamide derivatives **7a** and **7c** showed a significant decline in the cytotoxic activity (IC_{50} = 19.08 and 37.73 μM, respectively) compared to their analogs **3a** and **3c**.The 4-chloro derivative **7b** was nearly equipotent to **3b** (IC_{50} = 15.05 and 16.87 μM). Moreover, replacing oxygen by sulfur to obtain the corresponding carbothioamide analogs **7d-f** resulted in a significant enhancement in the cytotoxic activity (IC_{50} = 4.53, 7.18 and 22.40 μM, respectively) compared to their carboxamide derivatives **7a-c** which could be attributed to the increase in their lipophilicity.

Additionally, 1-phenyl/1-(4-methoxyphenyl)-3-styryl-4,5-dihydro-1*H*-pyrazolies **4a** and **4c** showed higher activity (IC_{50} = 6.61 and 6.14 μM, respectively) relative to their chloro derivative **4b** (IC_{50} = 10.20 μM). However, the presence of the styryl moiety at position 5 of the pyrazoline ring led to a decrease in the cytotoxic activity of the unsubstituted **8a** and 4-methoxyphenyl congener **8c** (IC_{50} = 12.95 and 20.48 μM, respectively). The chloro derivative **8b** revealed the highest activity in this series comparable to **4a-c** (IC_{50} = 3.93 μM). Finally, the 1-(4-methoxyphenyl)-5-phenyl-3-styrylpyrazoline derivative **4d** demonstrated a higher activity (IC_{50} = 6.66 μM) than its chloro **4e** and methoxy **4f** analogs (IC_{50} = 18.06 and 19.51 μM, respectively). Moreover, the positional isomer **8d** elicited a superior activity (IC_{50} = 3.79 μM) in comparison to **4d** concluding that compound **8d** was the most potent compound in comparison to both staurosporine and erlotinib. Notably, the condensed open products **8e** and **8f** showed a higher activity (IC_{50}= 13.28 and 6.00 μM, respectively) in comparison to the cyclized pyrazoline analogs **4e** and **4f** (IC_{50} = 18.06 and 19.51 μM, respectively).

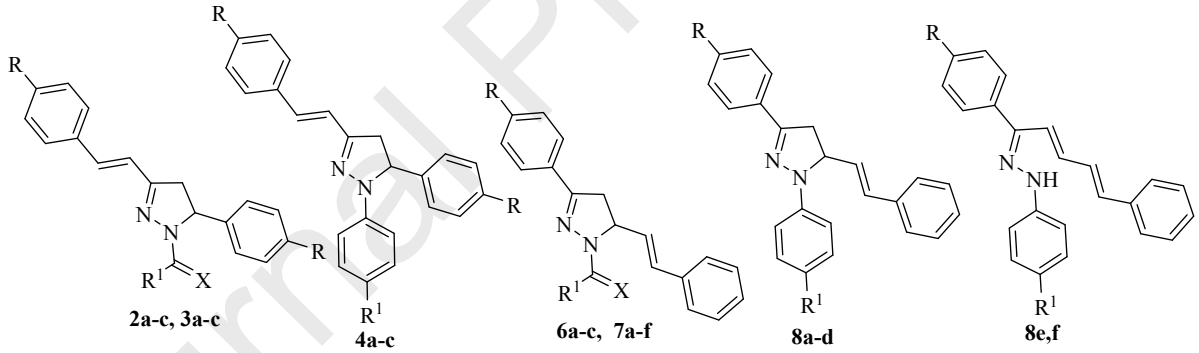
Regarding the safety of the tested compounds towards the normal cell line, compounds eliciting higher cytotoxicity against MCF-7 than staurosporine and erlotinib (compounds **2a**, **3a**, **3c**, **4a-d**, **6c**, **7d**, **7e**, **8b**, **8d** and **8f**) were selected to be screened for their cytotoxicity against normal fibroblasts (WI-38). The tested compounds were found to be more selective to MCF-7 cancer cell line than to the normal cell line WI-38 as they revealed cytotoxicity against normal fibroblasts WI-38 with IC_{50} of 11.24-54.22 μM and a selectivity index of 1.5-11.3. Therefore, they can be considered to be safe against the normal cell line.

2.3. EGFR inhibitory activity

In order to investigate the mechanism of action of the most active compounds **3c**, **6c**, **7d**, **8b** and **8d** that revealed the highest cytotoxicity against MCF-7 compared to erlotinib, they were tested for their EGFR inhibition using erlotinib as a reference standard. The results as IC_{50} (μM) are presented in Table 2.

From the obtained results, it can be noted that the EGFR inhibitory activity of the tested compounds was consistent with their cytotoxicity except for compound **8b** that exhibited moderate EGFR inhibition (IC_{50} = 1.39 μM) and promising cytotoxicity (IC_{50}= 3.93 μM). Therefore, it may act via another mechanism in addition to the EGFR inhibition. On the other hand, compounds **3c**, **6c**, **7d** and **8d** exhibited submicromolar inhibitory activity of EGFR (IC_{50} = 0.33, 0.46, 0.34 and 0.33 μM, respectively) comparable to erlotinib (IC_{50} = 0.23 μM) suggesting that they may exert their cytotoxic activity via EGFR inhibition.

Table 1. Cytotoxicity of the tested compounds against MCF-7 and WI38 cell lines (IC_{50}, μM) and selectivity index (SI)



Compd. No.	R	R^1	X	Cytotoxicity against (IC_{50}, μM)		Selectivity index (SI)*
				MCF-7	WI38	
2a	H	CH_3	O	6.92±0.24	17.37±0.77	2.5
2b	Cl	CH_3	O	29.19±1.58	NT**	NT**
2c	OCH_3	CH_3	O	17.47±0.07	NT**	NT**
3a	H	NH_2	O	10.59±0.38	25.39±1.13	2.3
3b	Cl	NH_2	O	16.87±0.95	NT**	NT**
3c	OCH_3	NH_2	O	4.46±0.11	16.23±0.65	3.6
4a	H	H	-	6.61±0.18	20.05±0.75	3.0
4b	Cl	H	-	10.20±0.44	15.72±0.71	1.5
4c	OCH_3	H	-	6.14±0.15	21.47±0.93	3.5
4d	H	OCH_3	-	6.66±0.25	20.61±1.16	3.1

4e	Cl	OCH_3	-	18.06±0.73	NT**	NT**
4f	OCH_3	OCH_3	-	19.51±1.23	NT**	NT**
6a	H	CH_3	O	22.01±0.93	NT**	NT**
6b	Cl	CH_3	O	31.61±1.19	NT**	NT**
6c	OCH_3	CH_3	O	4.33±0.06	49.13±2.18	11.3
7a	H	NH_2	O	19.082±0.83	NT**	NT**
7b	Cl	NH_2	O	15.051±0.71	NT**	NT**
7c	OCH_3	NH_2	O	37.73±1.42	NT**	NT**
7d	H	NH_2	S	4.53±0.28	39.10±2.37	8.6
7e	Cl	NH_2	S	7.18±0.37	54.22±3.29	7.5
7f	OCH_3	NH_2	S	22.40±1.52	NT**	NT**
8a	H	H	-	12.95±0.82	NT**	NT**
8b	Cl	H	-	3.93±0.07	11.24±0.52	2.9
8c	OCH_3	H	-	20.48±1.35	NT**	NT**
8d	H	OCH_3	-	3.79±0.04	25.56±1.59	6.7
8e	Cl	OCH_3	-	13.28±0.61	NT**	NT**
8f	OCH_3	OCH_3	-	6.01±0.22	28.02±2.18	4.7
Staurosporine	-	-	-	10.61±0.62	17.50±1.12	1.6
Erlotinib	-	-	-	4.74±0.14	NT**	NT**

*SI = activity of the tested compounds (IC_{50}) against normal cell line (WI-38)/ activity of the tested compounds (IC_{50}) against cancer cell line.
**NT: Not tested

2.4. Molecular docking study

This study was performed for compounds **3c**, **6c, 7d, 8b** and **8d** that were tested for their EGFR inhibitory activity in order to validate the obtained results and investigate their binding mode in the EGFR active site. Docking setup was first validated by self-docking of the co-crystallized ligand (Erlotinib) in the vicinity of the binding site of the enzyme with energy score (S) = −10.89 kcal/mol and RMSD of 1.47 Å and with the ability to reproduce all the key interactions accomplished by the co-crystallized ligand with the key amino acids in the binding site. H-bonding with Met769, through water mediated H-bonding with Thr766 and cation-π interaction with Lys721 (Figures 1 and 2 in supplementary materials).

The ability of the tested compounds to interact with the key amino acids in the binding site rationalizes their good activity as indicated by their binding pattern and docking score compared to that of Erlotinib (Figures 3-7 and table 2). The general binding pattern of the tested compounds **3c**, **6c, 7d** and **8d** is that they interact through hydrogen bonding with the key amino acid Met769. They also interact through hydrophobic interaction by

their phenyl moiety with the hydrophobic side chains of the amino acids Val702, Ala719, Met742 and Leu764.

Compound **3c**, showing the best experimental EGFR inhibitory activity with IC_{50} of 0.33 μM, showed the highest predicted binding affinity with docking score of −11.16 kcal/mol. Moreover, it showed a promising binding mode as it interacts through hydrogen bonding with the key amino acid Met769 by its pyrazoline nitrogen at position 2 and with the amino acid Gln767 by its carboxamide substituent at position 1 of the pyrazoline ring. Furthermore, its 4-methoxyphenyl moiety at position 5 of the pyrazoline ring enhances the binding affinity through its hydrophobic interaction with the hydrophobic side chains of the amino acids Val702, Ala719 and Leu820. On the other hand, although compound **8b** showed a hydrophobic interaction with the hydrophobic side chains of the amino acids Leu694, Val702, Met769 and Leu820 by its 4-chlorophenyl moiety at position 3 of the pyrazoline ring, its inability to interact with the key amino acid Met769 in the active site of EGFR (Figure 6) results in a low predicted docking score of −9.55 kcal/mol which is reflected inits moderate experimental EGFR inhibitory activity (IC_{50}= 1.39 μM). Thus, the obtained results confirmed that the binding mode was consistent with the EGFR inhibitory activity of the tested compounds.

Table 2: Docking energy scores (*S*) in kcal/mol of tested compounds, erlotinib, amino acids involved in binding and their EGFR inhibitory activity (IC_{50}μM)

Compound	Docking score (kcal/mol)	Amino acids involved in binding	EGFR IC_{50} (μM)
3c	−11.16	Met769 and Gln767	0.33
6c	−10.86	Met769 and Lys721	0.46
7d	−9.40	Met769, Gln767 and Thr766	0.34
8b	−9.55	-	1.39
8d	−10.52	Met769	0.33
Erlotinib	−10.89	Met769 and Thr766	0.23

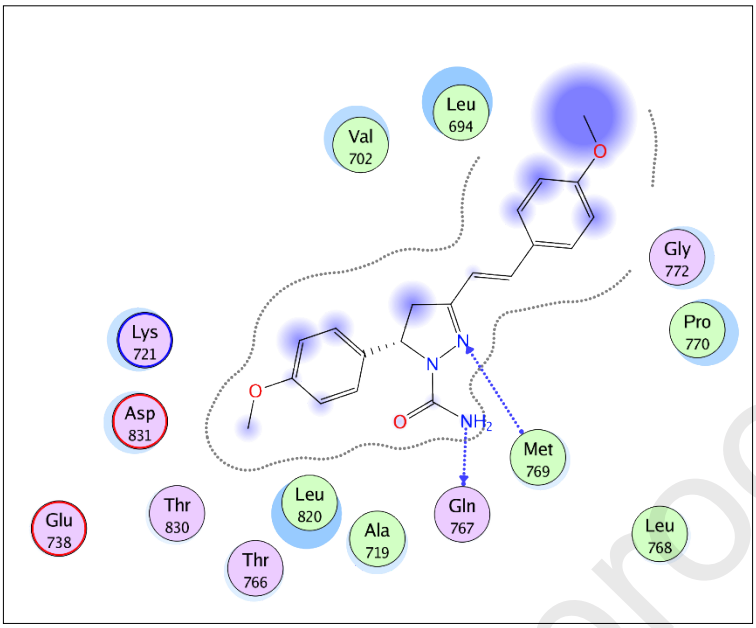


Figure 3. 2D diagram of compound **3c** in the EGFR binding site.

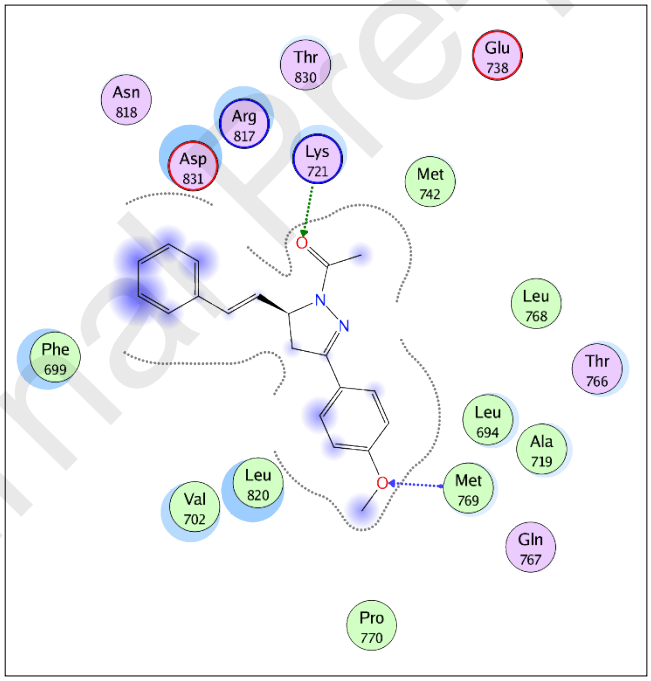


Figure 4. 2D diagram of compound **6c** in the EGFR binding site.

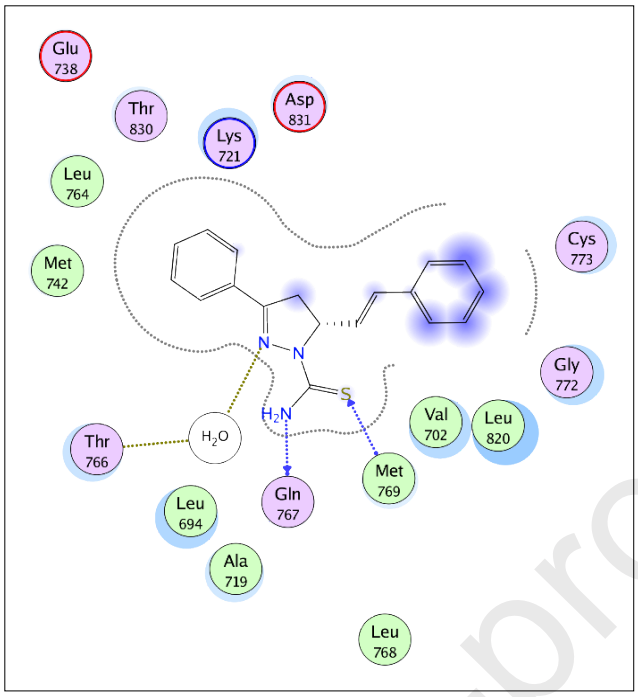


Figure 5. 2D diagram of compound **7d** in the EGFR binding site.

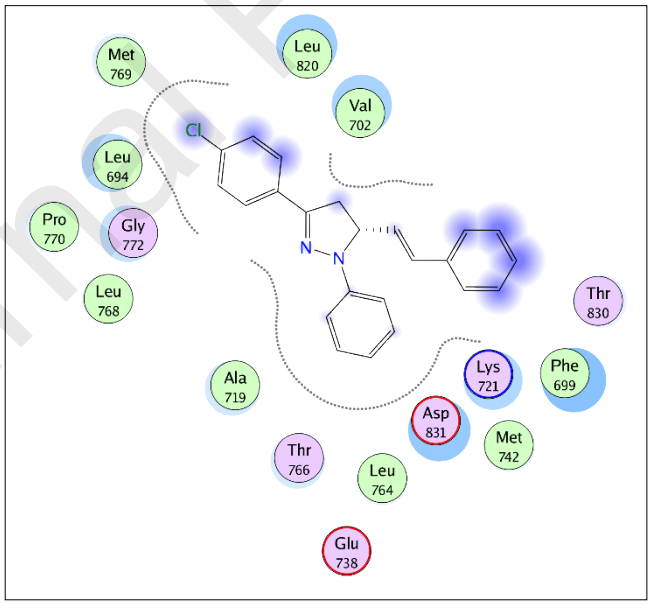


Figure 6. 2D diagram of compound **8b** in the EGFR binding site.

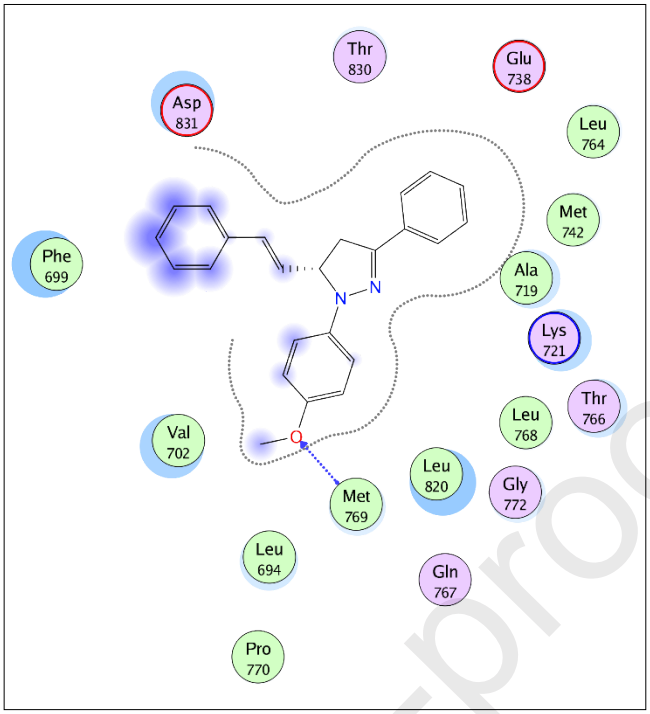


Figure 7. 2D diagram of compound **8d** in the EGFR binding site.

3. Conclusion

Twenty seven 1,3,5-trisubstituted pyrazolines were prepared by condensation reaction of the appropriate chalcone **1a-c** or **5a-c** with different hydrazine derivatives. Compounds **2a**, **3a**, **3c**, **4a-d**, **6c**, **7d**, **7e**, **8b**, **8d** and **8f** revealed higher activity than staurosporine and they were safe to the normal fibroblasts. Moreover, compounds **3c**, **6c**, **7d**, **8b** and **8d** exerted comparable cytotoxicity and EGFR inhibition relative to erlotinib except compound **8b** which revealed moderate EGFR concluding that it may exert its cytotoxicity via another mechanism in addition to EGFR inhibition. Molecular docking of compounds **3c**, **6c**, **7d**, **8b** and **8d** in the active site of EGFR validated the obtained results. Therefore, 1-substituted-5-aryl-3-styryl-4,5-dihydro-1*H*-pyrazolines and their positional isomers may represent interesting scaffolds for further optimization to obtain more promising cytotoxic agents with EGFR inhibitory activity.

4. Experimental

4.1. Chemistry

Melting points were detected using a Stuart SMP10 melting point apparatus. IR spectra (KBr) were performed on a Shimadzu FT-IR 8400S spectrophotometer. NMR spectra ($CDCl_3$) were carried out on a Bruker Ascend 400/R (^{1}H: 400 MHz, ^{13}C: 100 MHz) spectrometer. Mass spectra were measured on a GCMS-QP1000 EX spectrometer at 70 e.v. Elemental analyses were obtained utilizing FLASH 2000 CHNS/O analyzer, Thermo Scientific. Compounds **1a-c** [14-16], **2a-c** [19], **3a** [21], **4a-c** [22], **5a-c** [17,18], **6a-c, 8c** [20] and **8a** [14] were prepared according to the reported procedures and the data for the reported target compounds are present in the supplementary materials.

*4.1.1. General procedure for preparation of compounds **3a-c***

To a mixture of semicarbazide HCl (2.0 mmole) and the appropriate chalcones (**1a-c**) (2.0 mmole) in absolute ethanol (7 mL), sodium hydroxide (0.24 g, 6.0 mmole) was added. The reaction mixture was heated under reflux for 12 h till the reaction completed. Then, it was poured onto ice water with stirring, and the obtained precipitate was filtered, washed with water, dried and crystallized from ethanol.

*4.1.1.1. (E)-5-(4-Chlorophenyl)-3-(4-chlorostyryl)-4,5-dihydro-1H-pyrazole-1-carboxamide **(3b)***

Pale yellow crystals; yield (0.56 g, 93%); m.p. 139-140^{o}C; IR ν (cm^{-1}): 3450-3398 (NH_2), 3000-3050 (C-H aromatic), 2924 (C-H aliphatic), 1670 (C=O), 1573-1404 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ,* ppm): 1.79 (broad s, 2H, NH_2 exchanged with D_2O), 2.97 (dd, J = 5.20, 17.20 Hz, 1H, pyrazoline H_A), 3.62 (dd, J = 11.60, 17.20 Hz, 1H, pyrazoline H_M), 5.43 (dd, J = 5.20, 11.60 Hz, 1H, pyrazoline H_X), 6.65 (d, J = 16.40 Hz, 1H, olefinic H), 7.01 (d, J = 16.40 Hz, 1H, olefinic H), 7.16 (d, J = 8.40 Hz, 2H, aromatic H),7.30 (d, J = 8.40 Hz, 2H, aromatic H), 7.33 (d, J = 8.80 Hz, 2H, aromatic H), 7.38 (d, J = 8.40 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ,* ppm): 41.6 (CH_2 of pyrazoline), 59.5 (CH of pyrazoline), 121.0, 127.0, 128.1, 129.1, 129.2, 133.4, 134.2, 134.8, 135.4, 140.8, 152.6 (aromatic and olefinic carbons), 155.0 (C=O); Anal.

calcd. for $C_{18}H_{15}Cl_2N_3O$; C, 60.02; H, 4.20; N, 11.66; Found; C, 60.26; H, 4.37; N, 11.95.

4.1.1.2. (E)-5-(4-Methoxyphenyl)-3-(4-methoxystyryl)-4,5-dihydro-1H-pyrazole-1-carboxamide ***(3c)***

Pale yellow crystals; yield (0.55 g, 91%); m.p. 215-216°C; IR ν (cm^{-1}): 3498-3441 (NH_2), 3008 (C-H aromatic), 2912 (C-H aliphatic), 1660 (C=O), 1554-1415 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ,* ppm): 2.41 (broad s, 2H, NH_2 exchanged with D_2O), 3.02 (dd, *J* = 4.84, 17.16 Hz, 1H, pyrazoline H_A), 3.60 (dd, *J* = 11.84, 17.24 Hz, 1H, pyrazoline H_M), 3.79 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 5.43 (dd, *J* = 4.80, 11.80 Hz, 1H, pyrazoline H_X), 6.68 (d, *J* = 16.36 Hz, 1H, olefinic H), 6.86-6.96 (m, 5H, 4H aromatic H, 1H olefinic H), 7.18 (d, *J* = 8.56 Hz, 2H, aromatic H),7.41 (d, *J* = 8.60 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 41.7 (CH_2 of pyrazoline), 55.3 (OCH_3), 55.4 (OCH_3), 59.4 (CH of pyrazoline), 114.2, 114.3, 118.5, 126.6, 128.4, 128.6, 134.7, 136.2, 153.2, 155.1 (aromatic and olefinic carbons), 159.0 (C=O); MS m/z [%]: 351.12 [M^+, 72.31], 307.27 [100]; Anal. calcd. for $C_{20}H_{21}N_3O_3$; C, 68.36; H, 6.02; N, 11.96; Found; C, 68.59; H, 6.24; N, 12.20.

4.1.2. General procedure for preparation of compounds ***4a-f***

Phenyl hydrazine (0.24 g, 2.2 mmole) was added to the appropriate chalcones (**1a-c**) (2.0 mmole) in absolute ethanol (7 mL) and glacial acetic acid (1 mL). The reaction mixture was heated under reflux for 12 h till the reaction completed. Then, it was poured onto ice water with stirring; the obtained precipitate was filtered, washed with water, dried and crystallized from ethanol to obtain compounds (**4a-c**). Compounds **4d-f** were prepared similarly using equimolar amounts of 4-methoxyphenylhydrazine HCl and the appropriate chalcones (**1a-c**) (2.0 mmole) in absolute ethanol (7 mL).

4.1.2.1. (E)-1-(4-Methoxyphenyl)-5-phenyl-3-styryl-4,5-dihydro-1H-pyrazole ***(4d)***

Yellow crystals; yield (0.50 g, 66%); m.p. 120-121℃; IR ν (cm^{-1}): 3059-3001 (C-H aromatic), 2931-2904 (C-H aliphatic), 1604-1454 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 3.03 (dd, *J* = 8.00, 16.80 Hz, 1H, pyrazoline H_A), 3.68-3.747 (m, 4H, 1H,

pyrazoline H_M, 3H, OCH_3), 5.20 (dd, *J* = 8.00, 12.40 Hz, 1H, pyrazoline H_X), 6.54 (d, *J* = 16.40 Hz, 1H, olefinic H), 6.77 (d, *J* = 9.20 Hz, 2H, aromatic H), 6.98 (d, *J* = 8.80 Hz, 2H, aromatic H), 7.22-7.38 (m, 9H, 8H aromatic, 1H olefinic H), 7.46 (d, *J* = 7.20 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 45.4 (CH_2 of pyrazoline), 55.5 (OCH_3), 61.0 (CH of pyrazoline), 104.2, 114.5, 127.1, 127.2, 127.5, 127.9, 128.3, 128.4, 128.6, 128.7, 128.8, 129.4, 130.1, 134.4, 136.1, 160.0 (aromatic and olefinic carbons); Anal. calcd. for $C_{24}H_{22}N_2O$; C, 81.33; H, 6.26; N, 7.90; Found; C, 81.50; H, 6.53; N, 8.12.

4.1.2.2. (E)-5-(4-Chlorophenyl)-3-(4-chlorostyryl)-1-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole ***(4e)***

Yellow crystals; yield (0.60 g, 86%); m.p. 150-151℃; IR ν (cm^{-1}): 3043-3001 (C-H aromatic), 2927-2904 (C-H aliphatic), 1508-1465 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 2.96 (dd, *J* = 7.44, 16.64 Hz, 1H, pyrazoline H_A), 3.65-3.75 (m, 4H, 1H, pyrazoline H_M, 3H, OCH_3), 5.18 (dd, *J* = 8.00, 10.88 Hz, 1H, pyrazoline H_X), 6.47 (d, *J* = 16.24 Hz, 1H, olefinic H), 6.78 (d, *J* = 8.88 Hz, 2H, aromatic H), 6.94 (d, *J* = 8.88 Hz, 2H, aromatic H), 7.18 (d, *J* = 16.16 Hz, 1H, olefinic H), 7.25 (d, *J* = 8.28 Hz, 2H, aromatic H), 7.31-7.38 (m, 6H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 42.2 (CH_2 of pyrazoline), 55.6 (OCH_3), 64.6 (CH of pyrazoline), 104.6, 114.3, 114.5, 114.9, 120.5, 126.7, 127.4, 127.6, 127.7, 128.8, 128.9, 129.0, 129.3, 129.7, 129.9, 132.5, 133.4, 133.5, 134.6, 135.5, 138.4, 140.8, 143.2, 150.4, 159.2 (aromatic and olefinic carbons); MS m/z [%]: 423.53 [M^+, 6.98], 78.12 [100]; Anal. calcd. for $C_{24}H_{20}Cl_2N_2O$; C, 68.09; H, 4.76; N, 6.62; Found; C, 68.32; H, 4.98; N, 6.81.

4.1.2.3. (E)-1,5-Bis(4-methoxyphenyl)-3-(4-methoxystyryl)-4,5-dihydro-1H-pyrazole ***(4f)***

Yellow crystals; yield (0.50 g, 71%); m.p.173-174℃; IR ν (cm^{-1}): 3070-3001 (C-H aromatic), 2931-2848 (C-H aliphatic), 1604-1465 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 2.98-3.01 (m, 1H, pyrazoline H_A), 3.74-3.86 (m, 4H, 1H, pyrazoline H_M, 9H, 3 OCH_3), 5.18 (broad s, 1H, pyrazoline H_X), 6.50 (d, *J* = 15.76 Hz, 1H, olefinic H), 6.77 (d, *J* = 8.52 Hz, 2H, aromatic H), 6.87-6.96 (m, 6H, aromatic H), 7.10 (d, *J* = 16.08 Hz,

1H, olefinic H), 7.24 (d, *J* = 8.48 Hz, 2H, aromatic H), 7.40 (d, *J* = 7.16 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 42.5 (CH_2 of pyrazoline), 55.3 (OCH_3), 55.5 (OCH_3), 55.6 (OCH_3), 64.8 (CH of pyrazoline), 103.5, 114.0, 114.2, 114.3, 114.4, 119.7, 126.9, 127.2, 127.8, 128.1, 129.5, 130.0, 131.8, 134.6, 139.1, 144.6, 159.0, 159.6, 159.7, 159.9 (aromatic and olefinic carbons); Anal. calcd. for $C_{26}H_{26}N_2O_3$; C, 75.34; H, 6.32; N, 6.76; Found; C, 75.58; H, 6.59; N, 6.95.

*4.1.3. General procedure for preparation of compounds **7a-f***

To a mixture of semicarbazide HCl or thiosemicarbazide (2.0 mmole) and the appropriate chalcones (**5a-c**) (2.0 mmole) in absolute ethanol (7 mL), sodium hydroxide (0.24 g, 6.0 mmole) was added. The reaction mixture was heated under reflux for 12 h till the reaction completed. Then, it was poured onto ice water with stirring, and the obtained precipitate was filtered, washed with water, dried and crystallized from ethanol.

*4.1.3.1. (E)-3-Phenyl-5-styryl-4,5-dihydro-1H-pyrazole-1-carboxamide **(7a)***

Brown crystals; yield (0.50 g, 81%); m.p. 241-242^{o}C; IR ν (cm^{-1}): 3275-3205 (NH_2), 3055-3024 (C-H aromatic), 2920-2850 (C-H aliphatic), 1670 (C=O), 1573-1442 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 3.14 (dd, *J* = 5.00, 17.40 Hz, 1H, pyrazoline H_A), 3.59 (dd, *J* = 11.60, 17.36 Hz, 1H, pyrazoline H_M), 5.19-5.25 (m, 1H, pyrazoline H_X), 5.52 (broad s, 2H, NH_2 exchanged with D_2O), 6.29 (dd, *J* = 6.80, 15.80 Hz, 1H, olefinic H), 6.64 (d, *J* = 15.84 Hz, 1H, olefinic H), 7.21-7.31(m, 3H, aromatic H), 7.38-7.44 (m, 5H, aromatic H), 7.72-7.73 (m, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 40.0 (CH_2 of pyrazoline), 58.3 (CHof pyrazoline), 126.4, 126.7, 127.8, 127.9, 128.5, 128.7, 130.1, 131.1, 131.4, 136.3, 152.3 (aromatic and olefinic carbons), 155.6 (C=O); MS m/z [%]: 291.81 [M^+, 55.76], 211.60 [100]; Anal. calcd. for $C_{18}H_{17}N_3O$; C, 74.20; H, 5.88; N, 14.42; Found; C, 74.03; H, 6.09; N, 14.56.

*4.1.3.2. (E)-3-(4-Chlorophenyl)-5-styryl-4,5-dihydro-1H-pyrazole-1-carboxamide **(7b)***

White crystals; yield (0.50 g, 83%); m.p. 250-251^{o}C; IR ν (cm^{-1}): 3325-3263 (NH_2), 3051-3020 (C-H aromatic), 2931-2862 (C-H aliphatic), 1674 (C=O), 1589-1564 (C=C);

^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 3.12 (dd, J = 5.24, 17.40 Hz, 1H, pyrazoline H_A), 3.58 (dd, J = 11.60, 17.40 Hz, 1H, pyrazoline H_M), 5.20-5.26 (m, 1H, pyrazoline H_X), 5.41 (broad s, 2H, NH_2 exchanged with D_2O), 6.28 (dd, J = 6.88, 15.84 Hz, 1H, olefinic H), 6.63 (d, J = 15.84 Hz, 1H, olefinic H), 7.23 (t, J = 7.20 Hz, 1H, aromatic H), 7.31(d, J = 7.60 Hz, 2H, aromatic H), 7.40 (t, J = 8.36 Hz, 4H, aromatic H), 7.65 (d, J = 8.56 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 39.9 (CH_2 of pyrazoline), 58.5 (CH of pyrazoline), 126.7, 127.6, 127.7, 127.8, 128.5, 129.0, 130.0, 131.3, 136.0, 136.2, 151.1 (aromatic and olefinic carbons), 155.2 (C=O); MS m/z [%]: 327.18 [(M^++ 2), 3.04], 325.18 [M^+, 10.79], 44.15 [100]; Anal. calcd. for $C_{18}H_{16}ClN_3O$; C, 66.36; H, 4.95; N, 12.90; Found; C, 66.59; H, 5.12; N, 13.14.

4.1.3.3. (E)-3-(4-Methoxyphenyl)-5-styryl-4,5-dihydro-1H-pyrazole-1-carboxamide ***(7c)***

Off white crystals; yield (0.50 g, 83%); m.p. 205-206^{o}C; IR ν (cm^{-1}): 3278-3197 (NH_2), 3055-3028 (C-H aromatic), 2920-2846 (C-H aliphatic), 1681 (C=O), 1589-1516 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 3.11 (dd, J = 5.12, 17.32 Hz, 1H, pyrazoline H_A), 3.57 (dd, J = 11.52, 17.32 Hz, 1H, pyrazoline H_M), 3.87 (s, 3H, OCH_3), 5.17-5.23 (m, 1H, pyrazoline H_X), 5.41 (broad s, 2H, NH_2 exchanged with D_2O), 6.29 (dd, J = 6.84, 15.84 Hz, 1H, olefinic H), 6.63 (d, J = 15.84 Hz, 1H, olefinic H), 6.95 (d, J = 8.72 Hz, 2H, aromatic H), 7.23 (t, J = 7.16 Hz, 1H, aromatic H), 7.31 (d, J = 7.28 Hz, 2H, aromatic H), 7.39 (d, J = 7.36 Hz, 2H, aromatic H), 7.66 (d, J = 8.76 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 40.1 (CH_2 of pyrazoline), 55.4 (OCH_3), 58.1 (CH of pyrazoline), 114.1, 124.1, 126.6, 127.7, 128.0, 128.1, 128.5, 131.0, 136.4, 152.0, 161.1 (aromatic and olefinic carbons), 155.5 (C=O); Anal. calcd. for $C_{19}H_{19}N_3O_2$; C, 71.01; H, 5.96; N, 13.08; Found; C, 71.28; H, 6.19; N, 13.23.

4.1.3.4. (E)-3-Phenyl-5-styryl-4,5-dihydro-1H-pyrazole-1-carbothioamide ***(7d)***

Yellow crystals; yield (0.50 g, 77%); m.p. 240-241^{o}C; IR ν (cm^{-1}): 3495-3398 (NH_2), 3059-3028 (C-H aromatic), 2920-2850 (C-H aliphatic), 1577-1446 (C=C), 1246 (C=S); ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 3.16 (dd, J = 5.04, 17.36 Hz, 1H, pyrazoline H_A), 3.61 (dd, J = 11.52, 17.36 Hz, 1H, pyrazoline H_M), 5.21-5.24 (m, 1H, pyrazoline H_X), 5.41 (broad s, 2H, NH_2 exchanged with D_2O), 6.29 (dd, J = 6.84, 15.84 Hz, 1H, olefinic

H), 6.64 (d, *J* = 15.84 Hz, 1H, olefinic H), 7.23 (t, *J* = 7.20 Hz, 1H, aromatic H), 7.31 (d, *J* = 7.12 Hz, 2H, aromatic H),7.39 (d, *J* = 7.28 Hz, 2H, aromatic H), 7.44 (t, *J* = 7.24 Hz, 3H, aromatic H), 7.71-7.74 (m, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 40.1 (CH_2 of pyrazoline), 58.4 (CH of pyrazoline), 126.4, 126.7, 127.7, 127.8, 128.5, 128.7, 130.2, 131.3, 136.3 (aromatic and olefinic carbons), 172.2 (C=S); MS m/z [%]: 307.25 [M^+, 25.33], 115.84 [100]; Anal. calcd. for $C_{18}H_{17}N_3S$; C, 70.33; H, 5.57; N, 13.67; Found; C, 70.49; H, 5.79; N, 13.51.

4.1.3.5. (E)-3-(4-Chlorophenyl)-5-styryl-4,5-dihydro-1H-pyrazole-1-carbothioamide ***(7e)***

Brown crystals; yield (0.50 g, 79%); m.p. 200-201^{o}C; IR ν (cm^{-1}): 3471-3402 (NH_2), 3059-3028 (C-H aromatic), 2927-2854 (C-H aliphatic), 1593-1554 (C=C), 1253 (C=S); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 3.03 (dd, *J* = 4.96, 17.32 Hz, 1H, pyrazoline H_A), 3.49 (dd, *J* = 11.52, 17.28 Hz, 1H, pyrazoline H_M), 5.10-5.13 (m, 1H, pyrazoline H_X), 5.30 (broad s, 2H, NH_2 exchanged with D_2O), 6.18 (dd, *J* = 6.84, 15.84 Hz, 1H, olefinic H), 6.54 (d, *J* = 15.76 Hz, 1H, olefinic H), 6.98-7.21 (m, 4H, aromatic H), 7.30 (t, *J* = 8.60 Hz, 3H, aromatic H), 7.56 (d, *J* = 8.52 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 45.2 (CH_2 of pyrazoline), 65.2 (CH of pyrazoline), 116.8, 118.4, 126.7, 127.6, 127.8, 128.2, 128.5, 129.1, 129.5, 130.5. 135.2, 137.2 (aromatic and olefinic carbons), 172.3 (C=S); Anal. calcd. for $C_{18}H_{16}ClN_3S$; C, 63.24; H, 4.72; N, 12.29; Found; C, 63.52; H, 4.95; N, 12.53.

4.1.3.6. (E)-3-(4-Methoxyphenyl)-5-styryl-4,5-dihydro-1H-pyrazole-1-carbothioamide ***(7f)***

Brown crystals; yield (0.40 g, 63%); m.p. 205-206^{o}C; IR ν (cm^{-1}): 3460-3371 (NH_2), 3059-3001 (C-H aromatic), 2927-2839 (C-H aliphatic), 1600-1419 (C=C), 1253 (C=S); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 3.12 (dd, *J* = 5.12, 17.28 Hz, 1H, pyrazoline H_A), 3.58 (dd, *J* = 11.48, 17.28 Hz, 1H, pyrazoline H_M), 3.87 (s, 3H, OCH_3), 5.17-5.23 (m, 1H, pyrazoline H_X), 5.36 (broad s, 2H, NH_2 exchanged with D_2O), 6.28 (dd, *J* = 6.88, 15.88 Hz, 1H, olefinic H), 6.63 (d, *J* = 15.84 Hz, 1H, olefinic H), 6.95 (d, *J* = 8.80 Hz, 2H, aromatic H), 7.23 (t, *J* = 7.20 Hz, 1H, aromatic H), 7.31 (d, *J* = 7.20 Hz, 2H, aromatic H), 7.39 (d, *J* = 7.40 Hz, 2H, aromatic H), 7.66 (d, *J* = 8.76 Hz, 2H, aromatic

H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 40.3 (CH_2 of pyrazoline), 55.4 (OCH_3), 58.5 (CH of pyrazoline), 114.2, 123.5, 126.7, 127.0, 128.0, 128.2, 128.5, 132.0, 136.1, 154.0, 156.1 (aromatic and olefinic carbons), 172.5 (C=S); Anal. calcd. for $C_{19}H_{19}N_3OS$; C, 67.63; H, 5.68; N, 12.45; Found; C, 67.87; H, 5.80; N, 12.72.

4.1.4. General procedure for preparation of compounds ***8a-f***

Phenyl hydrazine (0.24 g, 2.2 mmole) was added to the appropriate chalcones (**5a-c**) (2.0 mmole) in absolute ethanol (7 mL) and glacial acetic acid (1 mL). The reaction mixture was heated under reflux for 12 h till the reaction completed. Then, it was poured onto ice water with stirring, the obtained precipitate was filtered, washed with water, dried and crystallized from ethanol to obtain compounds (**8a-c**). Compounds **8d-f** were prepared similarly using equimolar amounts of 4-methoxyphenylhydrazine HCl and the appropriate chalcones (**5a-c**) (2.0 mmole) in absolute ethanol (7 mL).

4.1.4.1. *(E)-3-(4-Chlorophenyl)-1-phenyl-5-styryl-4,5-dihydro-1H-pyrazole* ***(8b)***

Brown crystals; yield (0.50 g, 75%); m.p.100-101^{o}C; IR ν (cm^{-1}): 3059-3028 (C-H aromatic), 2924-2854 (C-H aliphatic), 1597-1492 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 3.12 (dd, J = 6.96, 16.84 Hz, 1H, pyrazoline H_A), 3.63 (dd, J = 12.00, 16.00 Hz, 1H, pyrazoline H_M), 5.13-5.16 (m, 1H, pyrazoline H_X), 6.15 (dd, J = 7.04, 16.00 Hz, 1H, olefinic H), 6.92 (d, J = 16.32 Hz, 1H, olefinic H), 7.06-7.60 (m, 10H, aromatic H), 7.87 (d, J = 8.44 Hz, 2H, aromatic H), 7.94 (d, J = 8.40 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 41.7 (CH_2 of pyrazoline), 61.3 (CH of pyrazoline), 113.6, 120.1, 124.6, 125.5, 126.4, 126.6, 126.7, 127.1, 128.0, 128.1, 128.3, 128.5, 128.6, 128.8, 129.0, 129.2, 129.3, 130.1, 132.8, 146.9 (aromatic and olefinic carbons); Anal. calcd. for $C_{23}H_{19}ClN_2$; C, 76.98; H, 5.34; N, 7.81; Found; C, 76.79; H, 5.60; N, 8.05.

4.1.4.2. *(E)-1-(4-Methoxyphenyl)-3-phenyl-5-styryl-4,5-dihydro-1H-pyrazole* ***(8d)***

Brown crystals; yield (0.40 g, 53%); m.p.105-106 ^{o}C; IR ν (cm^{-1}): 3055-3028 (C-H aromatic), 2954-2835 (C-H aliphatic), 1512-1462 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 3.13 (dd, J = 8.40, 16.80 Hz, 1H, pyrazoline H_A), 3.63 (dd, J = 11.60, 16.80 Hz, 1H, pyrazoline H_M), 3.78 (s, 3H, OCH_3), 4.80-4.87 (m, 1H, pyrazoline H_X), 6.36 (dd, J = 7.60, 16.00 Hz, 1H, olefinic H), 6.67 (d, J = 16.00 Hz, 1H, olefinic H), 6.87 (t, J = 8.80

Hz, 3H, aromatic H), 7.03 (d, *J* = 9.20 Hz, 2H, aromatic H), 7.15-7.54 (m, 5H, aromatic H), 7.74 (d, *J* = 7.20 Hz, 2H, aromatic H), 7.94 (d, *J* = 7.60 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 43.6 (CH_2 of pyrazoline), 55.6 (OCH_3), 62.3 (CH of pyrazoline), 100.6, 114.4, 114.6, 125.6, 126.4, 126.9, 127.3, 127.6, 128.4, 128.6, 128.8, 129.0, 129.2, 130.4, 130.9, 134.1, 135.9, 143.6, 150.7, 160.1 (aromatic and olefinic carbons); MS m/z [%]: 354.30 [M^+, 7.18], 77.11 [100]; Anal. calcd. for $C_{24}H_{22}N_2O$; C, 81.33; H, 6.26; N, 7.90; Found; C, 81.19; H, 6.53; N, 8.14.

4.1.4.3. (E)-1-((2E,4E)-1-(4-Chlorophenyl)-5-phenylpenta-2,4-dien-1-ylidene)-2-(4-methoxyphenyl)hydrazine ***(8e)***

Off white crystals; yield (0.50 g, 69%); m.p. 135-136^{o}C; IR ν (cm^{-1}): 3417 (NH), 3047-3020 (C-H aromatic), 2916-2850 (C-H aliphatic), 1516-1442 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 3.91 (s, 3H, OCH_3), 6.85 (d, *J*= 16.28 Hz, 1H, olefinic H), 6.95 (s, 1H, NH exchanged with D_2O), 7.06 (d, *J* = 8.42 Hz, 2H, aromatic H), 7.16 (d, *J* = 16.28 Hz, 1H, olefinic H), 7.30-7.45 (m, 9H, 2H olefinic H, 7H, aromatic H), 7.49 (d, *J* = 8.84 Hz, 2H, aromatic H), 7.87 (d, *J* = 8.52 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 55.6 (OCH_3), 100.5, 114.4, 115.5, 126.7, 127.0, 127.1, 127.4, 128.4, 128.8, 128.9, 129.4, 129.8, 131.6, 132.4, 133.6, 136.4, 142.9, 150.5, 159.5 (aromatic and olefinic carbons); Anal. calcd. for $C_{24}H_{21}ClN_2O$; C, 74.12; H, 5.44; N, 7.20; Found; C, 74.19; H, 5.67; N, 7.33.

4.1.4.4. (E)-1-(4-Methoxyphenyl)-2-((2E,4E)-1-(4-methoxyphenyl)-5-phenylpenta-2,4-dien-1-ylidene)hydrazine ***(8f)***

Brown crystals; yield (0.30 g, 42%); m.p.101-102^{o}C; IR ν (cm^{-1}): 3421 (NH), 3055-3001 (C-H aromatic), 2931-2835 (C-H aliphatic), 1600-1512 (C=C); ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*, ppm): 3.76 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 6.75 (d, *J* = 16.80 Hz, 2H, olefinic H), 6.81 (s, 1H, NH exchanged with D_2O), 6.88 (d, *J* = 8.80 Hz, 2H, aromatic H), 6.94 (d, *J* = 8.80 Hz, 2H, aromatic H), 7.04 (d, *J* = 16.40 Hz, 2H, olefinic H), 7.24-7.34 (m, 5H, aromatic H), 7.38 (d, *J* = 8.80 Hz, 2H, aromatic H), 7.75 (d, *J* = 8.80 Hz, 2H, aromatic H); ^{13}C NMR ($CDCl_3$, 100 MHz, *δ*, ppm): 55.5 (OCH_3), 55.6 (OCH_3), 100.1, 113.8, 114.0, 114.2, 114.4, 115.7, 126.6, 127.1, 127.3, 127.6, 128.3, 128.8, 128.9,

129.1, 130.7, 131.8, 132.1, 132.7, 136.2, 141.5, 142.6, 144.0, 151.5, 159.5 (aromatic and olefinic carbons); Anal. calcd. for $C_{25}H_{24}N_2O_2$; C, 78.10; H, 6.29; N, 7.29; Found; C, 78.23; H, 6.45; N, 7.50.

4.2. In vitro Cytotoxicity activity

The cytotoxic activity screening was performed at confirmatory diagnostic unit, VACSERA, Dokki, Cairo, Egypt. Culture medium was removed to a centrifuge tube, the cell layer rinsed with 0.25% (w/v) 0.53 mM EDTA solution to remove all traces of serum which contains Trypsin inhibitor, 2.0 to 3.0 mL of Trypsin EDTA solution was added to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Followed by adding 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting, then the cell suspension is transferred to the centrifuge tube with the medium and cells, centrifuge at approximately 125 X g for 5 to 10 minutes and the supernatant wad discarded. Moreover, the cell pellet was resuspended in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture vessels and incubate cultures at 37°C for 24 hrs. After treatment of cells with the serial concentrations of the compound to be tested incubation was carried out for 48 h at 37ºC, then the plates were examined under the inverted microscope and proceeded for the MTT assay. The cultures were removed from incubator into laminar flow hood or other sterile work area. Each vial of MTT [M-5655] reconstituted to be used with 3 ml of medium or balanced salt solution without phenol red and serum. Add reconstituted MTT in an amount equal to 10% of the culture medium volume, then the cultures were returned to incubator for 2-4 hours depending on cell type and maximum cell density. (An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity). Incubation times should be consistent when making comparisons. After the incubation period, cultures were removed from incubator and the resulting formazan crystals were dissolved by adding an amount of MTT Solubilization Solution [M-8910] equal to the original culture medium volume. Furthermore, gentle mixing in a gyratory shaker would enhance dissolution. Occasionally, especially in dense cultures, pipetting up and down [trituration] may be required to completely dissolve the MTT formazan crystals. Finally, the absorbance was measured spectrophotometrically at

a wavelength of 570 nm. The background absorbance of multiwell plates was measured at 690 nm and subtracted from the 450 nm measurement. Tests performed in multiwell plates can be read using the appropriate type of plate reader [23-25].

4.3. *EGFR inhibitory activity*

It was tested at the confirmatory diagnostic unit, VACSERA, Dokki, Cairo, Egypt. The master mixture (6 µL 5X Kinase Buffer + 1 µL ATP (500 µM) + 1 µL 50 X PTK substrate + 17 µL water) was prepared then, 25 µL to every well was added. 5 µL of Inhibitor solution of each well labeled as "Test Inhibitor" was added. However, for the"Positive Control" and "Blank", 5 µL of the same solution without inhibitor (Inhibitor buffer) was added. 3 ml of 1X Kinase Buffer by mixing 600 µL of 5X Kinase Buffer with 2400 µL water was prepared. So, 3 ml of 1X Kinase Buffer became sufficient for 100 reactions. To the wells designated as "Blank", 20 µl of 1X Kinase Buffer was added. EGFR enzyme on ice was thawed. Upon first thaw, briefly the tube containing enzyme was spun to recover full content of the tube. The amount of EGFR required for the assay and dilute enzyme to 1 ng/µL with 1X Kinase Buffer was calculated. Moreover, the remaining undiluted enzyme in aliquots was stored at -80°C. The reaction was initiated by adding 20 µL of diluted EGFR enzyme to the wells designated "Positive Control" and "Test Inhibitor Control", after that it was incubated at 30°C for 40 minutes. After the 40 minutes reaction, 50 µL of Kinase-Glo Max reagent was added to each well and the plate was covered with aluminum foil and incubated at room temperature for 15minutes. Luminescence was measured using the microplate reader.

4.4. Molecular docking study

All the molecular modeling studies were carried out using Molecular Operating Environment (MOE, 2010.10) software. All minimizations were performed with MOE until an RMSD gradient of 0.1 kcal·mol^{-1}Å^{-1} with MMFF94x force field and the partial charges were automatically calculated. The X-ray crystallographic structure of Epidermal Growth Factor Receptor (EGFR) co-crystallized with the 4-anilinoquinazoline derivative (Erlotinib) (PDB ID: 1M17) was downloaded from the protein data bank [27, 28]. Water molecules and ligands that are not involved in binding were removed. Next, the protein

was prepared for docking study using *Protonate 3D* protocol in MOE with default options. The co-crystalized ligand (Erlotinib) was used to define the binding site for docking. Triangle Matcher placement method and London dG scoring function were used for docking. Docking setup was first validated by self-docking of the co-crystallized ligand (Erlotinib) in the vicinity of the binding site of the enzyme with energy score (S) = −10.89 kcal/mol and RMSD of 1.47 Å and with the ability to reproduce all the key interactions accomplished by the co-crystallized ligand with the key amino acids in the binding site. H-bonding with Met769, through water mediated H-bonding with Thr766 and cation-π interaction with Lys721 (Figures 1 and 2 in supplementary materials). The validated setup was then used in predicting the ligands receptor interactions at the binding site for the compounds of interest.

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Some 1,3,5-Trisubstituted Pyrazoline Derivatives Targeting Breast Cancer: Design, Synthesis, Cytotoxic activity, EGFR inhibition and Molecular docking

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Highlights

- Some 1,3,5-trisubstituted-4,5-dihydro-1*H*-pyrazolines were prepared.

- They were screened for their anticancer activity against breast cell line MCF-7.

-Many compounds with promising anticancer activity were tested against normal fibroblasts.

- Promising cytotoxic compounds (**3c**, **6c**, **7d**, **8b** and **8d)** were tested for EGFR inhibition.

- Molecular docking in the EGFR active site confirmed the obtained activity.

Some 1,3,5-Trisubstituted Pyrazoline Derivatives Targeting Breast Cancer: Design, Synthesis, Cytotoxic activity, EGFR inhibition and Molecular docking

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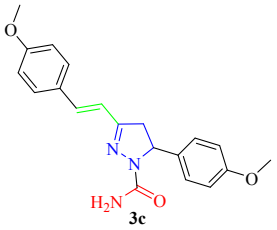
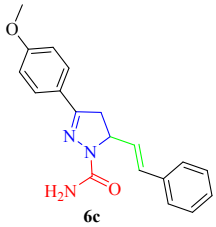


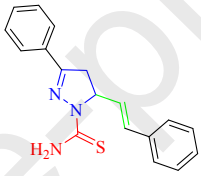
3c

IC_{50} on MCF-7 = 4.46 µM
IC_{50} on WI-38 = 16.23 µM
IC_{50} against EGFR = 0.33 µM



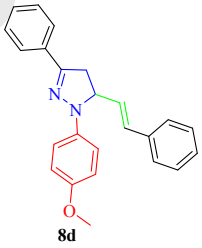
6c

IC_{50} on MCF-7 = 4.33 µM
IC_{50} on WI-38 = 49.13 µM
IC_{50} against EGFR = 0.46 µM



7d

IC_{50} on MCF-7 = 4.53 µM
IC_{50} on WI-38 = 39.10 µM
IC_{50} against EGFR = 0.34 µM



8d

IC_{50} on MCF-7 = 3.79 µM
IC_{50} on WI-38 = 25.56 µM
IC_{50} against EGFR = 0.33 µM

Declarations of interest: There are no interests to declare.