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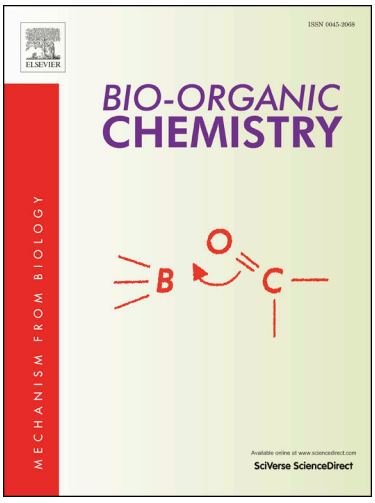

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Design, Synthesis And Biological Evaluation Of Some New 2-Pyrazoline Derivatives As Potential Anticancer Agents

Fatih Tok[a], Burçin İrem Abas[b], Özge Çevik[b], Bedia Koçyiğit-Kaymakçıoğlu[a,*]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

[b] Department of Biochemistry, School of Medicine, Aydin Adnan Menderes University, Aydın, Turkey

*Corresponding author:

Bedia Koçyiğit-Kaymakçıoğlu, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Marmara University, Istanbul 34668, Turkey. Tel: +90-216-4142963/1023 E-mail: bkaymakcioglu@marmara.edu.tr

Highlights

New chalcone and pyrazoline derivatives were designed and synthesized.

Anticancer activity of all synthesized compounds was investigated.

Their pharmacokinetic and drug-like properties were tested using swissadme online server.

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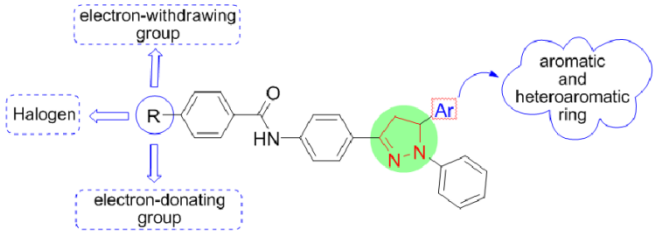

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Abstract

A new series of *N*-(4-(1-Phenyl-5-aryl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-substitutedbenzamide derivatives were designed and synthesized from new chalcone derivatives. All newly synthesized compounds were determined by using IR, ^{1}H-NMR, ^{13}C-NMR spectroscopic methods, elemental analysis and evaluated for their *in vitro* antiproliferative activities on HeLa, MCF-7, MKN-45 cancer cell lines and NIH-3T3 cell line using MTT assay. Expression of Bax and Bcl-2 proteins was detected by Western-blot analysis and caspase-3 enzyme activity was measured. Notably, compounds **1f** and **2f** showed a significant cytotoxic effect in all three cancer cells and did not display cytotoxicity on NIH-3T3 normal cells. (IC_{50} = 26.66±2.73 μM on HeLa, IC_{50} = 9.41±2.19 μM on MCF-7, IC_{50} = 5.17±3.54 μM on MKN-45 for **1f**. IC_{50} = 17.96±3.34 μM on HeLa, IC_{50} = 0.69±0.13 μM on MCF-7, IC_{50} = 0.88±0.16 μM on MKN-45 for **2f**.) Moreover, **1f** and **2f** upregulated protein expression level of Bax and downregulated protein expression level of Bcl-2 in cells. Similarly, caspase-3 activity was increased in cells via **1f** and **2f.** It can be concluded that **1f** and **2f** activated apoptosis by inducing mitochondrial apoptotic proteins in HeLa, MCF-7, MKN-45. This could be potentially new anti-cancer derivatives and used to contribute to new therapeutic development.

Keywords: Pyrazoline; anticancer; caspase activity; apoptosis; chalcone; MTT assay; HeLa.

1 .Introduction

It is predicted that cancer incidence will reach from 18.1 million to 29.5 million, cancer mortality from 9.6 million to 16.3 million burdens worldwide from the current estimates in 2018 up until 2040 by World Health Organisation [1]. Therefore chemotherapeutic agents play an important role in the cancer therapy. Many anticancer drugs have potential disadvantages such as reduced bioavailability, toxicity, drug-resistance, few drug targets, higher cost. Medicinal chemists aim to develop new anticancer drugs with better properties [2].

Pyrazolines are very important examples of biologically active five-membered ring compounds in the area of drug design [3]. *N-N* bond linkage of the pyrazoline ring is regarded as the key factor in their biological activities. Living organisms establish these bonds more difficulty, therefore *N-N* bonds are less available in natural compounds [4]. Pyrazolines have a variety of

biological activities such as antibacterial, antifungal, antiviral, analgesic, antidepressant, anti-inflammatory and antitumor activities [5-11]. Many drug molecules carrying pyrazole and pyrazoline ring with different activities are currently available in the market. For example, antipyrine, ramifenazone, morazone, celecoxib, fezolamine and tepoxalin is adducible [12-14] (Figure 1).

Figure 1

In this study, we aimed the synthesis of new pyrazoline derivatives from new chalcones and these synthesized compounds were screened potential anticancer effects on HeLa (human cervical adenocarcinoma), MCF-7 (human breast adenocarcinoma), MKN-45 (human gastric carcinoma) cancer cell lines. The tumor selectivity of the synthesized compounds was also determined on NIH-3T3 mouse embryonic fibroblast cell line using MTT assay.

2. Results and discussion

2.1. Chemistry

New pyrazoline derivatives were synthesized in three steps and synthetic route for the preparation of target compounds as described in Figure 2. In the first step, amide derivatives were synthesized with 4-aminoacetophenone and different benzoyl chloride in chloroform. Then, new chalcone derivatives were prepared according to Claisen-Schmidt condensation by *N*-(4-acetylphenyl)-4-substitutedbenzamide with different aromatic aldehydes in aqueous ethanolic KOH. Finally, new pyrazoline derivatives were obtained with chalcones and phenylhydrazine hydrochloride in acetic acid (Figure 2). The structures of chalcone and pyrazoline (**1a-s, 2a-s**) were established on the basis IR, ^{1}H-NMR, ^{13}C-NMR spectral data and elemental analysis.

Figure 2

IR spectra of chalcone derivatives afforded C=C stretching (1550-1599 cm^{-1}), amide group N-H stretching (3281-3418 cm^{-1}) and C=O stretching bands (1646-1680 cm^{-1}). In the ^{1}H-NMR spectrum of chalcone derivatives, olefinic protons were observed with aromatic protons as multiplet in the range of 6.17-8.40 ppm.

IR spectra of pyrazoline derivatives afforded C=N stretching (1589-1624 cm^{-1}), amide group N-H stretching (3290-3392 cm^{-1}) and C=C stretching bands (1579-1597 cm^{-1}). The ^{1}H-NMR spectrum of pyrazoline ring showed three different signals as doublet of doublet attributed Ha, Hb and Hx protons due to the ABX spin system in the structures. The Ha, Hb and Hx protons

resonated at 3.07-3.38 ppm (J_{ab}: 17.24-17.88 Hz), 3.77-3.98 ppm (J_{ax}: 5.98-6.41 Hz) and 5.41-5.68 ppm (J_{bx}: 11.90-12.36 Hz), respectively. NH protons of amide groups appeared as a singlet peak at 10.29-10.71 ppm. All other aliphatic and aromatic protons were observed at expected regions. In ^{13}C-NMR spectra, the C3, C4 and C5 carbons of the pyrazoline ring were found at 147.5-148.7 ppm, 41.5-43.5 ppm and 57.1-63.5 ppm, respectively. Amide C=O and chalcone C=O peaks appeared at 161.7-166.3 ppm and 186.7-188.7 ppm, respectively. The values of elemental analysis were within ± 0.4% of the theoretical values.

2.2. Biology

2.2.1. Cytotoxicity of the compound

The cytotoxicity of the synthesized new chalcone and pyrazoline derivatives was assessed in three cancer cells lines: cervix (HeLa), breast (MCF-7) and gastric (MKN-45). Cancer cells were incubated with these newly synthesized compounds (0-1000 μM) at different concentrations for 24 hours. Cell viability was measured as a colorimetric assay based on mitochondrial dehydrogenase activities changes and into a colored formazan production. According to our results, the compounds exhibit the highest concentrations of toxicity. (Table 1). However, the compounds were found to be different IC_{50} values in cells when compared with having an effect below 50 μM concentration between cells. Therefore, compounds with the appropriate IC_{50} value must exhibit less than 50 μM, a clinically significant concentration, to become a drug candidate. Potential anticancer drug candidates should also be cytotoxic to cancer cells but harmless to normal cells. Toxicity profiles of the newly synthesized chalcone and pyrazoline derivatives were tested against NIH-3T3 normal mouse fibroblast cells and were found not to harm healthy cells. Cell survival was performed with compound IC_{50} values of **1f**, **2f**, **1h**, **2h**, **1m** and **2m** on HELA cells and **1f, 2f, 1i, 2i, 1r** and **2r** on MKN-45 cells and **1d, 2d, 1f** and **2f** on MCF-7 cells for 6h, 24 h and 48h. Compound **1f** and **2f** appeared to have the same anti-survival effect in all three cells (Figure 3a-c). Compound **1f** and **2f** selected from synthesized chalcone and pyrazoline derivatives were treated with cells and changes in the cells were observed under a light microscope. The selected compound **1f** and **2f** had an effect on the cells and cellular contractions occurred in HELA, MKN-45 and MCF7 cells. At the same time, the interconnection of the cells with each other decreased and exhibited a more rounded morphology rather than spreading adhesive cells (Figure 3d). Looking at the data, the compounds **1f** and **2f** were found to show strong anticancer activity and were potentially potential drug candidates.

Figure 3

Heterocyclic compounds are extremely important for the development of active molecules. Biological molecules such as DNA, RNA bear some heterocyclic ring in their major skeleton. These rings interact enzyme or receptor easily due to the ability to form hydrogen bond acceptors and donors. Therefore the probability of showing strong activity increases. Compounds **2f**, **2l** and **2s** contain pyrazoline ring as well as furan ring demonstrated strong cytotoxic effect on cancer cells. Moreover these compounds **2f**, **2l** and **2s** showed higher activity than chalcone analogs such as **1f, 1l** and **1s**.

Table 1

2.2.2. Cell apoptosis of the compounds

Apoptosis occurs in the cell in two ways, one is called extrinsic and the other is intrinsic ways, apoptosis is determined depending on the initiator signal. The extrinsic pathway is initiated in the cell membrane through death receptors. On the other hand, the intrinsic pathway is controlled through the Bcl-2 protein family and affects the permeability of the mitochondrial outer membrane. Both ways It is controlled by cysteine proteases specific to aspartic acid residues and its common enzyme caspase-3 in two ways. To investigate the effects of compounds **1f** and **2f** on HeLa, MCF7, MKN-45 cell apoptosis, these cells were treated with IC_{50} μM values for 24h and then analysis of caspase-3 activity and mitochondrial apoptotic proteins were performed. The caspase-3 activity showed a significant increase (compare to control) in the **1f** and **2f** compounds treated HeLa, MCF7, MKN-45 cells (Figure 4a). Western blotting was performed to evaluate the mitochondrial apoptosis expression levels of the Bax, Bcl-2 and GAPDH (control) proteins. As expected, the proapoptotic Bax levels were increased in the 1f and 2f compounds and the antiapoptotic Bcl-2 levels were decreased in the **1f** and **2f** compounds treated HeLa, MCF7, MKN-45 cells (Figure 4b-e). Importantly, alterations in the relative levels of Bax and Bcl-2 are important in determining whether cells will undergo apoptosis. The ratio of Bax/Bcl-2 protein expression levels in cells treated with compounds **1f** and **2f** was significantly increased in HeLa, MCF7, MKN-45 cells (Figure 4f). The present findings suggest that the cell apoptosis induced by treatment with **1f** and **2f** is dependent on alterations in the expression of Bcl-2 family proteins and caspase-3 activity, and is associated with the mitochondrial pathway in HeLa, MCF7, MKN-45 cells.

Figure 4

2.3. Physicochemical properties and pharmacokinetic profile predictions

Prediction of physicochemical and pharmacokinetic properties of the compounds is very important for the development of new drug candidates. If these properties of molecules are not good enough, it can not succeed in clinical candidate drugs for the treatment of illness. Bioavailability of synthesized compounds can be tested with determining druglike properties by using the Lipinski rule of five and the Veber rule. Compounds should have at least three of the following properties according to Lipinski rule: molecular weight should be lower than 500, less than 5 logP, number of hydrogen bond donors below 5 and number of hydrogen bond acceptors below 10. Furthermore, the number of rotatable bonds should be lower than the maximum value of 10 and the Polar Surface Area should be less than the maximum value of 140 Å2 according to Veber rule [15-17]. The screening results are presented in Table 2. These values demonstrate that all the synthesized compounds are not violating the Lipinski and Veber rules. Especially compound **1f** and **2f**, the most promising agent, exhibited hopeful bioavailability score which was defined as good permeability. Compound **1f** and **2f** were moderately soluble and showed without any violations of other drug-like properties Egan and Ghose rules (Table 3).

Table 2

Table 3

Swissadme program provides important information about pharmacokinetic properties (such as gastrointestinal absorption, brain access and P-glycoprotein) of molecules. BOILED-Egg server predicts these pharmacokinetics by using wLogP and TPSA. The yellow region (BBB) shows molecules which are for a high probability of passively permeated through the blood brain barrier. The white region (HIA) shows molecules that are for a high probability of passively absorbed by the gastrointestinal tract. However, molecules on the gray region have low absorption and limited brain access. Blue dots (PGP+) are for molecules predicted to be flowed out from the central nervous system by the P-glycoprotein. Red dots (PGP-) are for molecules predicted not to be effluated from the central nervous system by the P-glycoprotein [15]. It was predicted that compound **1j** from chalcones and **2**j from pyrazolines had low gastrointestinal absorption and brain penetration because both molecules carried steric hindrance from bulky substituents such as nitro groups on aromatic rings. All compounds except **1j** and **2j** exhibited good bioavailability. Compound **1f** and **2f**, the most promising agents, displayed high blood-brain permeability and gastrointestinal absorption. Compound **1f** was not subject to active

efflux (red dot), however compound **2f** was pumped-out from the brain (blue dot) easily because of being a P-glycoprotein substrate. Compound **1f** and **2f** can be potential candidates for drug discovery because of their good drug-likeness and pharmacokinetic properties The boiled egg predictive models of all compounds were given in Figure 5.

Figure 5

3. Conclusion

In this article, we synthesized new chalcone and pyrazoline derivatives which have exhibited cytotoxic activities against HeLa, MCF7, MKN-45 cancer cell lines, and NIH-3T3 normal cell. Among them, compounds **2l** and **2s** containing pyrazole and furan ring demonstrated the highest cytotoxic activity on MCF7 and MKN-45 cancer cells. (IC_{50} = 0.06±0.04 μM on MCF-7, IC_{50} = 0.02±0.01 μM on MKN-45 for **2l**. IC_{50} = 0.16±0.04 μM on MCF-7, IC_{50} = 0.02±0.01 μM on MKN-45 for **2s**.) On the other hand, **1f** and **2f** having furan moiety showed strong anticancer activity against all cancer cells with good selectivity. (IC_{50} = 26.66±2.73 μM on HeLa, IC_{50} = 9.41±2.19 μM on MCF-7, IC_{50} = 5.17±3.54 μM on MKN-45 for **1f**. IC_{50} = 17.96±3.34 μM on HeLa, IC_{50} = 0.69±0.13 μM on MCF-7, IC_{50} = 0.88±0.16 μM on MKN-45 for **2f**.) Cytotoxic effect of **1f** and **2f** on NIH-3T3 normal cell did not observe. We observed that compound **1f** and **2f** were a potent inducer of apoptosis in HeLa, MCF7, MKN-45 cells and caused an alteration in the induction of mitochondrial apoptotic events. At the same time, the promising compound **1f** and **2f** decreased the level of Bcl-2 and increased the level of Bax, which was consistent with the result of caspase-3 enzyme activity analysis in inducing apoptosis. Therefore, compounds **1f** and **2f** can be potential anticancer agents with further investigation.

4. Experimental

4.1. Chemistry

Chemicals and solvents were purchased from Sigma Aldrich (St. Louis, MO, USA), Merck(Darmstadt, Germany). The progress of reactions was monitored via thin layer chromatography (TLC), performed on commercially available silica gel (Kieselgel 60, F254) coated aluminium sheets (Merck) by using petroleum ether:ethyl acetate (10:90) as solvent system. The visualization on TLC was done under ultra-violet (UV) light (λ= 254 nm). Melting points were determined by the Schmelzpunktbestimmer SMP II apparatus. Infrared spectra were recorded on a Shimadzu FTIR 8400 S Spectrometer. Proton nuclear magnetic resonance (NMR) (400 MHz) and carbon NMR (150 MHz) spectra were obtained (DMSO-d_6) were run

on a Bruker ACP 200 spectrometer (Bruker Corp., Billerica, MA, USA). It was used deuterodimethylsulfoxide (DMSO-d_6) as the solvent and tetramethylsilane (TMS) as an internal standard. Elemental analysis was determined by CHNS-932 (LECO).

4.1.1. General procedure of amide synthesis

Firstly, 1mmol *4-aminoacetophenone* was dissolved in 20 mL chloroform. 1mmol Substitutedbenzoyl chloride was added to the reaction liquid and it was stirred and refluxed in a water bath over a period of 8 h. After evaporation of the solvent, amide derivatives were washed with distilled water and filtered [18].

4.1.2. General procedure of chalcone synthesis

1mmol *N-(4-acetylphenyl)-4-substitutedbenzamide* and different aromatic aldehydes were dissolved in ethanol. %40 KOH (1mL) was added to the reaction liquid. It was stirred on room temperature for 10 h and poured into ice-cold water. The precipitated product was washed with water, filtered and recrystallized from methanol [19].

*4.1.2.1. N-(4-Cinnamoylphenyl)-4-fluorobenzamide (**1a**)*

Yield 75%; white solid; m.p. 201.2-202.0ºC; IR (ν_{max}, cm^{-1}): 3362 (N-H), 3061 (=C-H), 1654 (C=O), 1585, 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.36-8.21 (m, 15H, CH=CH and Ar-H), 10.59 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 188.0, 165.5, 144.1, 143.9, 135.2, 133.1, 131.5, 131.1, 131.0, 129.4, 129.2, 122.5, 120.1, 119.9, 116.1, 115.9, 115.7; Anal. calcd. for $C_{22}H_{16}FNO_2$: C, 76.51; H, 4.67; N, 4.06; Found: C, 76.47; H, 4.65; N, 4.09%.

*4.1.2.2. 4-Fluoro-N-(4-(3-(4-fluorophenyl)acryloyl)phenyl)benzamide (**1b**)*

Yield 77%; white solid; m.p. 222.3-222.9ºC; IR (ν_{max}, cm^{-1}): 3369 (N-H), 3086 (=C-H), 1656 (C=O), 1593, 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.27-8.20 (m, 14H, CH=CH and Ar-H), 10.61 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 188.0, 165.5, 164.6, 163.9, 144.2, 142.6, 133.1, 132.5, 131.9, 131.6, 131.5, 131.1, 130.2, 129.7, 122.3, 120.1, 119.9, 116.4, 115.9; Anal. calcd. for $C_{22}H_{15}F_2NO_2$: C, 72.72; H, 4.16; N, 3.85; Found: C, 72.62; H, 4.14; N, 3.82%.

*4.1.2.3. N-(4-(3-(4-Chlorophenyl)acryloyl)phenyl)-4-fluorobenzamide (**1c**)*

Yield 72%; yellow solid; m.p. 164.5-165.4ºC; IR (ν_{max}, cm^{-1}): 3281 (N-H), 3030 (=C-H), 1647 (C=O), 1597, 1587 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.36-8.21 (m, 14H, CH=CH and

Ar-H), 10.59 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ186.8, 164.4, 162.8, 143.2, 141.3, 134.4, 133.1, 131.9, 130.4, 130.0, 129.9, 128.7, 128.3, 122.1, 119.0, 118.9, 114.9, 114.7; Anal. calcd. for $C_{22}H_{15}ClFNO_2$: C, 69.57; H, 3.98; N, 3.69; Found: C, 69.65; H, 3.95; N, 3.72%.

*4.1.2.4. 4-Fluoro-N-(4-(3-(4-nitrophenyl)acryloyl)phenyl)benzamide (**1d**)*

Yield 70%; yellow solid; m.p. 236.7-237.5ºC; IR (ν_{max}, cm^{-1}): 3363 (N-H), 3080 (=C-H), 1666 (C=O), 1589 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.37-8.30 (m, 14H, CH=CH and Ar-H), 10.63 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 188.7, 164.5, 162.8, 147.4, 143.6, 140.7, 139.9, 131.6, 130.4, 130.1, 129.3, 125.4, 123.3 119.0, 114.9, 114.7; Anal. calcd. for $C_{22}H_{15}FN_2O_4$: C, 67.69; H, 3.87; N, 7.18; Found: C, 67.77; H, 3.85; N, 7.23%.

*4.1.2.5. 4-Fluoro-N-(4-(3-p-tolylacryloyl)phenyl)benzamide (**1e**)*

Yield 85%; yellow solid; m.p. 203.9-204.5ºC; IR (ν_{max}, cm^{-1}): 3404 (N-H), 3032 (=C-H), 1666 (C=O), 1591, 1566 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ2.35 (s, 3H, CH_3), 7.26-8.12 (m, 14H, CH=CH and Ar-H), 10.49 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ186.9, 164.4, 162.8, 143.1, 142.9, 139.9, 132.1, 131.5, 130.4, 130.1, 129.1, 128.9, 120.3, 119.0, 118.9, 114.9, 114.8, 20.5; Anal. calcd. for $C_{23}H_{18}FNO_2$: C, 76.86; H, 5.05; N, 3.90; Found: C, 76.74; H, 5.06; N, 3.89%.

*4.1.2.6. 4-Fluoro-N-(4-(3-(furan-2-yl)acryloyl)phenyl)benzamide (**1f**)*

Yield 83%; yellow solid; m.p. 180.3-181.1ºC; IR (ν_{max}, cm^{-1}): 3366 (N-H), 3095 (=C-H), 1651 (C=O), 1599, 1581 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ7.40-8.19 (m, 13H, CH=CH and Ar-H), 10.61 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{20}H_{14}FNO_3$: C, 71.64; H, 4.21; N, 4.18; Found: C, 71.77; H, 4.19; N, 4.20%.

*4.1.2.7. N-(4-Cinnamoylphenyl)-4-nitrobenzamide (**1g**)*

Yield 75%; yellow solid; m.p. 235.6-236.3ºC; IR (ν_{max}, cm^{-1}): 3335 (N-H), 3076 (=C-H), 1672, 1649 (C=O), 1591, 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.45-8.37 (m, 15H, CH=CH and Ar-H), 10.81 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{22}H_{16}N_2O_4$: C, 70.96; H, 4.33; N, 7.52; Found: C, 70.87; H, 4.30; N, 7.57%.

*4.1.2.8. N-(4-(3-(4-Fluorophenyl)acryloyl)phenyl)-4-nitrobenzamide (**1h**)*

Yield 78%; yellow solid; m.p. 204.5-205.3ºC; IR (ν_{max}, cm^{-1}): 3418 (N-H), 3093 (=C-H), 1680, 1660 (C=O), 1587 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.27-8.39 (m, 14H, CH=CH and Ar-H), 10.85 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{22}H_{15}FN_2O_4$: C, 67.69; H, 3.87; N, 7.18; Found: C, 67.75; H, 3.84; N, 7.23%.

*4.1.2.9. N-(4-(3-(4-Chlorophenyl)acryloyl)phenyl)-4-nitrobenzamide (**1i**)*

Yield 79%; yellow solid; m.p. 186.6-187.3ºC; IR (ν_{max}, cm^{-1}): 3381 (N-H), 3080 (=C-H), 1670, 1646 (C=O), 1586, 1566 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.17-8.38 (m, 14H, CH=CH and Ar-H), 10.91 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{22}H_{15}ClN_2O_4$: C, 64.95; H, 3.72; N, 6.89; Found: C, 64.87; H, 3.69; N, 6.94%.

*4.1.2.10. 4-Nitro-N-(4-(3-(4-nitrophenyl)acryloyl)phenyl)benzamide (**1j**)*

Yield 73%; yellow solid; m.p. 226.7-227.4ºC; IR (ν_{max}, cm^{-1}): 3381 (N-H), 3093 (=C-H), 1672, 1655 (C=O), 1575 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.80-8.40 (m, 14H, CH=CH and Ar-H), 10.92 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{22}H_{15}N_3O_6$: C, 63.31; H, 3.62; N, 10.07; Found: C, 63.19; H, 3.59; N, 10.02%.

*4.1.2.11. 4-Nitro-N-(4-(3-p-tolylacryloyl)phenyl)benzamide (**1k**)*

Yield 74%; yellow solid; m.p. 201.2-202.1ºC; IR (ν_{max}, cm^{-1}): 3379 (N-H), 3093 (=C-H), 2983, 2887 (C-H), 1672, 1651 (C=O), 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 7.27-8.39 (m, 14H, CH=CH and Ar-H), 10.88 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{23}H_{18}N_2O_4$: C, 71.49; H, 4.70; N, 7.25; Found: C, 71.32; H, 4.67; N, 7.26%.

*4.1.2.12. N-(4-(3-(furan-2-yl)acryloyl)phenyl)-4-nitrobenzamide (**1l**)*

Yield 81%; yellow solid; m.p. 212.3-212.9ºC; IR (ν_{max}, cm^{-1}): 3377 (N-H), 3093 (=C-H), 1674, 1656 (C=O), 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.69-8.40 (m, 13H, CH=CH and Ar-H), 10.89 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.5, 165.5, 163.9, 151.7, 146.6, 144.0, 133.1, 131.5, 131.1, 130.5, 129.9, 120.1, 119.1, 117.3, 115.9, 113.6; Anal. calcd. for $C_{20}H_{14}N_2O_5$: C, 66.30; H, 3.89; N, 7.73; Found: C, 66.41; H, 3.88; N, 7.76%.

*4.1.2.13. N-(4-Cinnamoylphenyl)-4-methylbenzamide (**1m**)*

Yield 82%; white solid; m.p. 210.8-211.2ºC; IR (ν_{max}, cm^{-1}): 3371 (N-H), 3056 (=C-H), 2968, 2904 (C-H), 1666, 1651 (C=O), 1593, 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.40 (s, 3H, CH_3), 7.35-8.21 (m, 15H, CH=CH and Ar-H), 10.51 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 188.1, 166.3, 144.4, 143.8, 142.4, 135.2, 132.9, 130.9, 129.7, 129.4, 129.2, 128.4, 122.4, 120.0, 119.9, 21.51; Anal. calcd. for $C_{23}H_{19}NO_2$: C, 80.92; H, 5.61; N, 4.10; Found: C, 81.01; H, 5.59; N, 4.13%.

*4.1.2.14. N-(4-(3-(4-Fluorophenyl)acryloyl)phenyl)-4-methylbenzamide (**1n**)*

Yield 80%; white solid; m.p. 181.5-182.3ºC; IR (ν_{max}, cm^{-1}): 3373 (N-H), 3082 (=C-H), 2970, 2912 (C-H), 1672, 1651 (C=O), 1593, 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 7.01-8.21 (m, 14H, CH=CH and Ar-H), 10.49 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.9, 161.7, 144.3, 144.1, 143.8, 142.6, 133.2, 132.9, 131.9, 131.6, 130.2, 130.1, 129.7, 129.3, 128.4, 127.9, 122.3, 119.9, 116.5, 114.8, 21.51; Anal. calcd. for $C_{23}H_{18}FNO_2$: C, 76.86; H, 5.05; N, 3.90; Found: C, 76.72; H, 5.08; N, 3.88%.

*4.1.2.15. N-(4-(3-(4-Chlorophenyl)acryloyl)phenyl)-4-methylbenzamide (**1o**)*

Yield 77%; white solid; m.p. 208.3-208.9ºC; IR (ν_{max}, cm^{-1}): 3355 (N-H), 3086 (=C-H), 2978, 2904 (C-H), 1651 (C=O), 1597, 1572 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.38 (s, 3H, CH_3), 7.33-8.20 (m, 14H, CH=CH and Ar-H), 10.47 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 186.8, 165.2, 143.3, 141.4, 134.3, 133.2, 131.7, 129.9, 128.7, 128.3, 127.3, 122.1, 118.9, 20.4; Anal. calcd. for $C_{23}H_{18}ClNO_2$: C, 73.50; H, 4.83; N, 3.73; Found: C, 73.60; H, 4.85; N, 3.75%.

*4.1.2.16. 4-Methyl-N-(4-(3-(4-nitrophenyl)acryloyl)phenyl)benzamide (**1p**)*

Yield 85%; white solid; m.p. 200.9-201.4ºC; IR (ν_{max}, cm^{-1}): 3348 (N-H), 3086 (=C-H), 2978, 2906 (C-H), 1658 (C=O), 1587, 1573 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 7.34-8.30 (m, 14H, CH=CH and Ar-H), 10.51 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 186.7, 165.2, 147.4, 143.7, 141.4, 140.7, 139.9, 131.4, 129.3, 128.6, 128.3, 127.3, 125.4, 123.3, 118.9, 20.45; Anal. calcd. for $C_{23}H_{18}N_2O_4$: C, 71.49; H, 4.70; N, 7.25; Found: C, 71.62; H, 4.71; N, 7.23%.

*4.1.2.17. 4-Methyl-N-(4-(3-p-tolylacryloyl)phenyl)benzamide (**1r**)*

Yield 82%; white solid; m.p. 155.3-155.9ºC; IR (ν_{max}, cm^{-1}): 3352 (N-H), 3032 (=C-H), 2982 (C-H), 1688, 1649 (C=O), 1591, 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 7.69-8.20 (m, 14H, CH=CH and Ar-H), 10.49 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 188.0, 166.2, 144.2, 143.9, 142.4, 141.0, 133.0, 132.5, 132.1, 130.1, 129.7, 128.3, 121.3, 119.9, 26.9, 21.5; Anal. calcd. for $C_{24}H_{21}NO_2$: C, 81.10; H, 5.96; N, 3.94; Found: C, 80.97; H, 5.97; N, 3.93%.

*4.1.2.18. N-(4-(3-(furan-2-yl)acryloyl)phenyl)-4-methylbenzamide (**1s**)*

Yield 82%; white solid; m.p. 187.6-188.2ºC; IR (ν_{max}, cm^{-1}): 3331 (N-H), 3095 (=C-H), 2980 (C-H), 1680, 1651 (C=O), 1597, 1550 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 6.69-8.12 (m, 13H, CH=CH and Ar-H), 10.50 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 187.5, 166.2, 151.7, 146.5, 144.2, 142.5, 132.9, 130.4, 129.9, 129.4, 128.3, 120.0, 119.8, 117.2, 113.57, 21.51; Anal. calcd. for $C_{21}H_{17}NO_3$: C, 76.12; H, 5.17; N, 4.23; Found: C, 76.21; H, 5.15; N, 4.25%.

4.1.3. General procedure of pyrazoline synthesis

10 mmol chalcone derivatives and 10 mmol phenylhydrazine hydrochloride were stirred in glacial acetic acid (10 mL) and heated under reflux for 12 h. The precipitated product was washed with water, filtered and recrystallized from ethanol [20].

*4.1.3.1. N-(4-(1,5-diphenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-fluorobenzamide (**2a**)*

Yield 75%; yellow solid; m.p. 250.8-251.2ºC; IR (ν_{max}, cm^{-1}): 3336 (N-H), 3063 (=C-H), 1651 (C=O), 1589 (C=N), 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.08 (dd, *Jax* 6.36 Hz, *Jab* 17.40 Hz, 1H, Ha), 3.92 (dd, *Jbx* 12.16 Hz, *Jab* 17.39 Hz, 1H, Hb), 5.46 (dd, *Jax* 6.34 Hz, *Jbx* 12.12 Hz, 1H, Hx), 6.68-8.07 (m, 18H, Ar-H), 10.40 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 165.4, 163.7, 147.5, 144.8, 143.1, 140.0, 131.7, 130.9, 129.4, 129.3, 128.0, 127.8, 126.6, 126.3, 120.6, 118.9, 115.9, 115.7, 113.3, 63.5, 43.5; Anal. calcd. for $C_{28}H_{22}FN_3O$: C, 77.22; H, 5.09; N, 9.65; Found: C, 77.25; H, 5.10; N, 9.67%.

*4.1.3.2. 4-Fluoro-N-(4-(5-(4-fluorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2b**)*

Yield 78%; yellow solid; m.p. 270.8-271.5ºC; IR (ν_{max}, cm^{-1}): 3336 (N-H), 3049 (=C-H), 1651 (C=O), 1593 (C=N), 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.10 (dd, *Jax* 6.26 Hz, *Jab* 17.42 Hz, 1H, Ha), 3.90 (dd, *Jbx* 12.15 Hz, *Jab* 17.41 Hz, 1H, Hb), 5.49 (dd, *Jax* 6.21 Hz,

Jbx 12.10 Hz, 1H, Hx), 6.72-8.06 (m, 17H, Ar-H), 10.39 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 165.4, 164.9, 163.7, 162.6, 160.9, 147.5, 144.7, 140.1, 139.2, 131.6, 130.9, 129.3, 128.3, 128.0, 126.7, 120.6, 119.0, 116.3, 115.9, 113.3, 62.8, 43.4; Anal. calcd. for $C_{28}H_{21}F_2N_3O$: C, 74.16; H, 4.67; N, 9.27; Found: C, 74.33; H, 4.64; N, 9.25%.

*4.1.3.3. N-(4-(5-(4-Chlorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-fluorobenzamide (**2c**)*

Yield 81%; yellow solid; m.p. 303.1-303.9ºC; IR (ν_{max}, cm^{-1}): 3331 (N-H), 3049 (=C-H), 1651 (C=O), 1595 (C=N), 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.12 (dd, *Jax* 6.23 Hz, *Jab* 17.44 Hz, 1H, Ha), 3.91 (dd, *Jbx* 12.16 Hz, *Jab* 17.41 Hz, 1H, Hb), 5.50 (dd, *Jax* 6.20 Hz, *Jbx* 12.14 Hz, 1H, Hx), 6.70-8.07 (m, 17H, Ar-H), 10.40 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 165.4, 164.9, 163.7, 147.6, 144.6, 142.0, 140.1, 132.3, 131.6, 130.9, 129.4, 128.3, 127.9, 126.7, 120.6, 119.0, 115.9, 113.3, 62.8, 43.3; Anal. calcd. for $C_{28}H_{21}ClFN_3O$: C, 71.56; H, 4.50; N, 8.94; Found: C, 71.71; H, 4.48; N, 8.98%.

*4.1.3.4. 4-Fluoro-N-(4-(5-(4-nitrophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2d**)*

Yield 83%; red solid; m.p. 277.2-278.1ºC; IR (ν_{max}, cm^{-1}): 3336 (N-H), 3082 (=C-H), 1649 (C=O), 1593 (C=N), 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.14 (dd, *Jax* 6.28 Hz, *Jab* 17.49 Hz, 1H, Ha), 3.98 (dd, *Jbx* 12.28 Hz, *Jab* 17.51 Hz, 1H, Hb), 5.68 (dd, *Jax* 6.26 Hz, *Jbx* 12.28 Hz, 1H, Hx), 6.71-8.30 (m, 17H, Ar-H), 10.41 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 165.5, 164.9, 163.7, 150.6, 148.7, 144.4, 141.7, 140.9, 132.7, 131.2, 131.0, 130.5, 129.5, 127.9, 126.7, 128.8, 124.2, 120.1, 119.3, 115.7, 113.3, 62.8, 42.5; Anal. calcd. for $C_{28}H_{21}FN_4O_3$: C, 69.99; H, 4.41; N, 11.66; Found: C, 70.11; H, 4.40; N, 11.68%.

*4.1.3.5. 4-Fluoro-N-(4-(1-phenyl-5-p-tolyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2e**)*

Yield 84%; yellow solid; m.p. 265.5-266.3ºC; IR (ν_{max}, cm^{-1}): 3321 (N-H), 3084 (=C-H), 2989, 2895 (C-H), 1649 (C=O), 1591 (C=N), 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H, CH_3), 3.11 (dd, *Jax* 6.20 Hz, *Jab* 17.85 Hz, 1H, Ha), 3.89 (dd, *Jbx* 11.90 Hz, *Jab* 17.81 Hz, 1H, Hb), 5.42 (dd, *Jax* 6.25 Hz, *Jbx* 11.92 Hz, 1H, Hx), 6.69-8.08 (m, 17H, Ar-H), 10.41 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 165.4, 163.7, 147.5, 144.8, 140.1, 137.0, 131.7, 130.9, 130.0, 129.2, 128.1, 126.2, 120.6, 118.8, 115.9, 115.7, 113.3, 63.3, 43.5, 21.1; Anal. calcd. for $C_{29}H_{24}FN_3O$: C, 77.49; H, 5.38; N, 9.35; Found: C, 77.57; H, 5.39; N, 9.40%.

*4.1.3.6. 4-Fluoro-N-(4-(5-(furan-2-yl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2f**)*

Yield 77%; yellow solid; m.p. 272.7-273.6ºC; IR (ν_{max}, cm^{-1}): 3338 (N-H), 3057 (=C-H), 1654 (C=O), 1589 (C=N), 1579 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.37 (dd, *Jax* 5.98 Hz, *Jab* 17.28 Hz, 1H, Ha), 3.78 (dd, *Jbx* 12.16 Hz, *Jab* 17.28 Hz, 1H, Hb), 5.58 (dd, *Jax* 5.98 Hz, *Jbx* 12.17 Hz, 1H, Hx), 6.37-8.08 (m, 16H, Ar-H), 10.41 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 165.4, 163.7, 153.7, 147.9, 144.9, 143.3, 140.1, 131.6, 130.8, 129.3, 127.9, 126.8, 120.7, 119.3, 116.0, 115.8, 115.6, 113.6, 111.0, 108.3, 57.1, 42.5; Anal. calcd. for $C_{26}H_{20}FN_3O_2$: C, 73.40; H, 4.74; N, 9.88; Found: C, 73.29; H, 4.78; N, 9.91%.

*4.1.3.7. N-(4-(1,5-Diphenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-nitrobenzamide (**2g**)*

Yield 84%; red solid; m.p. 220.1-220.8ºC; IR (ν_{max}, cm^{-1}): 3331 (N-H), 3059 (=C-H), 1691 (C=O), 1622 (C=N), 1589 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.11 (dd, *Jax* 6.41 Hz, *Jab* 17.39 Hz, 1H, Ha), 3.91 (dd, *Jbx* 12.19 Hz, *Jab* 17.39 Hz, 1H, Hb), 5.46 (dd, *Jax* 6.37 Hz, *Jbx* 12.16 Hz, 1H, Hx), 6.68-8.38 (m, 18H, Ar-H), 10.68 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 164.3, 149.6, 147.4, 144.7, 143.0, 140.8, 139.6, 129.7, 129.3, 128.5, 127.8, 126.7, 124.0, 120.7, 118.9, 113.3, 63.5, 43.5; Anal. calcd. for $C_{28}H_{22}N_4O_3$: C, 72.71; H, 4.79; N, 12.11; Found: C, 72.66; H, 4.80; N, 12.14%

*4.1.3.8. N-(4-(5-(4-Fluorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-nitrobenzamide (**2h**)*

Yield 80%; red solid; m.p. 226.3-226.9ºC; IR (ν_{max}, cm^{-1}): 3319 (N-H), 3057 (=C-H), 1691 (C=O), 1620 (C=N), 1597 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.11 (dd, *Jax* 6.28 Hz, *Jab* 17.86 Hz, 1H, Ha), 3.90 (dd, *Jbx* 12.36 Hz, *Jab* 17.88 Hz, 1H, Hb), 5.50 (dd, *Jax* 6.28 Hz, *Jbx* 12.36 Hz, 1H, Hx), 6.69-8.38 (m, 17H, Ar-H), 10.69 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ 164.3, 162.6, 161.0, 149.6, 147.4, 144.6, 140.8 139.6, 139.1, 129.7, 128.4, 128.3, 126.7, 124.0, 120.7, 119.0, 116.1, 113.4, 62.8, 43.4; Anal. calcd. for $C_{28}H_{21}FN_4O_3$: C, 69.99; H, 4.41; N, 11.66; Found: C, 69.89; H, 4.43; N, 11.69%.

*4.1.3.9. N-(4-(5-(4-Chlorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-nitrobenzamide (**2i**)*

Yield 78%; red solid; m.p. 232.8-233.5ºC; IR (ν_{max}, cm^{-1}): 3344 (N-H), 3085 (=C-H), 1697 (C=O), 1620 (C=N), 1589 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.09 (dd, *Jax* 6.20 Hz,

Jab 17.40 Hz, 1H, Ha), 3.92 (dd, *Jbx* 12.17 Hz, *Jab* 17.41 Hz, 1H, Hb), 5.51 (dd, *Jax* 6.19 Hz, *Jbx* 12.15 Hz, 1H, Hx), 6.70-8.38 (m, 17H, Ar-H), 10.70 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ164.3, 149.6, 147.5, 144.5, 141.9, 140.8, 139.6, 132.3, 129.7, 129.4, 128.4, 126.7, 124.0, 120.7, 119.1, 113.3, 62.8, 43.3; Anal. calcd. for $C_{28}H_{21}ClN_4O_3$: C, 67.67; H, 4.26; N, 11.27; Found: C, 67.78; H, 4.22; N, 11.25%.

*4.1.3.10. 4-Nitro-N-(4-(5-(4-nitrophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2j**)*

Yield 80%; red solid; m.p. 207.4-208.2ºC; IR (ν_{max}, cm^{-1}): 3309 (N-H), 3095 (=C-H), 1687 (C=O), 1622 (C=N), 1593 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.14 (dd, *Jax* 6.33 Hz, *Jab* 17.44 Hz, 1H, Ha), 3.97 (dd, *Jbx* 12.24 Hz, *Jab* 17.44 Hz, 1H, Hb), 5.66 (dd, *Jax* 6.32 Hz, *Jbx* 12.24 Hz, 1H, Hx), 6.71-8.37 (m, 17H, Ar-H), 10.71 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ164.4, 150.5, 149.6, 147.6, 144.4, 140.8, 139.8, 129.8, 129.7, 128.1, 127.9, 126.9, 124.8, 124.3, 124.0, 120.8, 119.4, 113.3, 62.9, 43.3; Anal. calcd. for $C_{28}H_{21}N_5O_5$: C, 66.27; H, 4.17; N, 13.80; Found: C, 66.37; H, 4.20; N, 13.75%.

*4.1.3.11. 4-Nitro-N-(4-(1-phenyl-5-p-tolyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2k**)*

Yield 82%; red solid; m.p. 240.1-240.8ºC; IR (ν_{max}, cm^{-1}): 3348 (N-H), 3095 (=C-H), 2987, 2906 (C-H), 1697 (C=O), 1620 (C=N), 1595 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H, CH_3), 3.07 (dd, *Jax* 6.37 Hz, *Jab* 17.38 Hz, 1H, Ha), 3.88 (dd, *Jbx* 12.15 Hz, *Jab* 17.38 Hz, 1H, Hb), 5.41 (dd, *Jax* 6.34 Hz, *Jbx* 12.12 Hz, 1H, Hx), 6.67-8.38 (m, 17H, Ar-H), 10.68 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ164.3, 149.6, 147.3, 144.7, 140.8, 139.5, 137.0, 130.0, 129.7, 128.5, 126.8, 126.2, 124.0, 120.8, 118.9, 113.3, 63.4, 42.5, 21.1; Anal. calcd. for $C_{29}H_{24}N_4O_3$: C, 73.09; H, 5.08; N, 11.76; Found: C, 73.15; H, 5.08; N, 11.73%.

*4.1.3.12. N-(4-(5-(furan-2-yl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-nitrobenzamide (**2l**)*

Yield 82%; red solid; m.p. 209.4-210.1ºC; IR (ν_{max}, cm^{-1}): 3290 (N-H), 3084 (=C-H), 1687 (C=O), 1622 (C=N), 1591 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.38 (dd, *Jax* 5.98 Hz, *Jab* 17.28 Hz, 1H, Ha), 3.78 (dd, *Jbx* 12.19 Hz, *Jab* 17.28 Hz, 1H, Hb), 5.60 (dd, *Jax* 5.98 Hz, *Jbx* 12.17 Hz, 1H, Hx), 6.38-8.39 (m, 16H, Ar-H), 10.71 (s, 1H, NH); ^{13}C-NMR (150 MHz,DMSO-d_6): δ164.4, 153.7, 149.6, 147.8, 144.8, 143.2, 140.9, 139.6, 129.7, 128.4, 126.7,

124.0, 120.8, 119.2, 113.6, 110.9, 108.2, 57.1, 41.5; Anal. calcd. for $C_{26}H_{20}N_4O_4$: C, 69.02; H, 4.46; N, 12.38; Found: C, 69.15; H, 4.49; N, 12.24%.

*4.1.3.13. N-(4-(1,5-Diphenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-methylbenzamide (**2m**)*

Yield 83%; yellow solid; m.p. 217.7-218.5ºC; IR (ν_{max}, cm^{-1}): 3338 (N-H), 3072 (=C-H), 2987, 2906 (C-H), 1656 (C=O), 1624 (C=N), 1593 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 3.11 (dd, *Jax* 6.37 Hz, *Jab* 17.39 Hz, 1H, Ha), 3.91 (dd, *Jbx* 12.17 Hz, *Jab* 17.39 Hz, 1H, Hb), 5.46 (dd, *Jax* 6.39 Hz, *Jbx* 12.15 Hz, 1H, Hx), 6.68-7.90 (m, 18H, Ar-H), 10.29 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.8, 147.5, 144.8, 143.1, 142.2, 140.2, 132.3, 129.4, 129.3, 128.2, 127.8, 126.6, 126.3, 120.5, 118.8, 113.3, 63.5, 43.5, 21.5; Anal. calcd. for $C_{29}H_{25}N_3O$: C, 80.72; H, 5.84; N, 9.74; Found: C, 80.75; H, 5.85; N, 9.77%.

*4.1.3.14. N-(4-(5-(4-Fluorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-methylbenzamide (**2n**)*

Yield 75%; yellow solid; m.p. 212.8-213.6ºC; IR (ν_{max}, cm^{-1}): 3296 (N-H), 3063 (=C-H), 2960, 2895 (C-H), 1691 (C=O), 1622 (C=N), 1593 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 3.09 (dd, *Jax* 6.30 Hz, *Jab* 17.40 Hz, 1H, Ha), 3.90 (dd, *Jbx* 12.08 Hz, *Jab* 17.39 Hz, 1H, Hb), 5.46 (dd, *Jax* 6.26 Hz, *Jbx* 12.09 Hz, 1H, Hx), 6.69-7.96 (m, 17H, Ar-H), 10.29 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.8, 147.6, 144.8, 144.7, 142.2, 140.2, 132.8, 129.4, 129.4, 128.4, 127.5, 126.6, 126.3, 120.6, 118.8, 113.3, 63.0, 43.5, 21.5; Anal. calcd. for $C_{29}H_{24}FN_3O$: C, 77.49; H, 5.38; N, 9.35; Found: C, 77.61; H, 5.41; N, 9.32%.

*4.1.3.15. N-(4-(5-(4-Chlorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-methylbenzamide (**2o**)*

Yield 73%; yellow solid; m.p. 216.7-217.7ºC; IR (ν_{max}, cm^{-1}): 3387 (N-H), 3095 (=C-H), 2974, 2906 (C-H), 1687 (C=O), 1620 (C=N), 1595 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 3.12 (dd, *Jax* 6.21 Hz, *Jab* 17.42 Hz, 1H, Ha), 3.91 (dd, *Jbx* 12.15 Hz, *Jab* 17.41 Hz, 1H, Hb), 5.56 (dd, *Jax* 6.21 Hz, *Jbx* 12.11 Hz, 1H, Hx), 6.70-7.90 (m, 17H, Ar-H), 10.29 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.8, 147.6, 144.6, 142.2, 142.0, 140.3, 132.3, 130.6, 129.6, 129.4, 128.3, 127.7, 126.7, 120.6, 119.0, 113.3, 62.8, 43.3, 21.5; Anal. calcd. for $C_{29}H_{24}ClN_3O$: C, 74.75; H, 5.19; N, 9.02; Found: C, 75.69; H, 5.17; N, 9.05%.

*4.1.3.16. 4-Methyl-N-(4-(5-(4-nitrophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2p**)*

Yield 77%; red solid; m.p. 169.9-170.2ºC; IR (ν_{max}, cm^{-1}): 3308 (N-H), 3072 (=C-H), 2960, 2906 (C-H), 1691 (C=O), 1622 (C=N), 1595 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H, CH_3), 3.17 (dd, *Jax* 6.31 Hz, *Jab* 17.47 Hz, 1H, Ha), 3.98 (dd, *Jbx* 12.24 Hz, *Jab* 17.50 Hz, 1H, Hb), 5.67 (dd, *Jax* 6.34 Hz, *Jbx* 12.22 Hz, 1H, Hx), 6.71-8.23 (m, 17H, Ar-H), 10.30 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.8, 150.6, 147.8, 147.3, 144.5, 142.2, 140.4, 132.3, 129.7, 129.4, 128.3, 127.8, 126.8, 124.7, 120.5, 119.8, 119.3, 113.3, 62.8, 43.1, 21.5; Anal. calcd. for $C_{29}H_{24}N_4O_3$: C, 73.09; H, 5.08; N, 11.76; Found: C, 72.99; H, 5.05; N, 11.78%.

*4.1.3.17. 4-Methyl-N-(4-(1-phenyl-5-p-tolyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzamide (**2r**)*

Yield 76%; yellow solid; m.p. 208.3-209.3ºC; IR (ν_{max}, cm^{-1}): 3392 (N-H), 3088 (=C-H), 2974, 2916 (C-H), 1691 (C=O), 1621 (C=N), 1597 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 3.08 (dd, *Jax* 6.32 Hz, *Jab* 17.36 Hz, 1H, Ha), 3.89 (dd, *Jbx* 12.14 Hz, *Jab* 17.37 Hz, 1H, Hb), 5.41 (dd, *Jax* 6.32 Hz, *Jbx* 12.14 Hz, 1H, Hx), 6.67-7.90 (m, 17H, Ar-H), 10.29 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.8, 147.5, 144.8, 142.2, 140.2, 140.1, 137.0, 132.3, 130.0, 129.7, 129.4, 128.3, 127.9, 126.6, 120.6, 119.8, 118.8, 113.3, 63.3, 43.5, 21.4, 21.1; Anal. calcd. for $C_{30}H_{27}N_3O$: C, 80.87; H, 6.11; N, 9.43; Found: C, 80.91; H, 6.15; N, 9.41%.

*4.1.3.18. N-(4-(5-(furan-2-yl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)-4-methylbenzamide (**2s**)*

Yield 80%; red solid; m.p. 246.2-246.9ºC; IR (ν_{max}, cm^{-1}): 3362 (N-H), 3095 (=C-H), 2982, 2916 (C-H), 1655 (C=O), 1622 (C=N), 1595 (C=C); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 3.11 (dd, *Jax* 5.98 Hz, *Jab* 17.24 Hz, 1H, Ha), 3.77 (dd, *Jbx* 12.12 Hz, *Jab* 17.24 Hz, 1H, Hb), 5.58 (dd, *Jax* 5.98 Hz, *Jbx* 12.16 Hz, 1H, Hx), 6.37-7.91 (m, 16H, Ar-H), 10.30 (s, 1H, NH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.8, 153.7, 147.9, 144.9, 143.2, 142.2, 140.3, 132.3, 129.4, 129.2, 128.2, 127.8, 126.6, 120.6, 119.2, 113.6, 110.9, 108.2, 57.1, 43.5, 21.5; Anal. calcd. for $C_{27}H_{23}N_3O_2$: C, 76.94; H, 5.50; N, 9.97; Found: C, 77.05; H, 5.49; N, 10.00%.

4.2. Anticancer activity

The changes in the anti-cancer activities of the synthesized new chalcone and pyrazoline derivatives were tested on three human cancer cell lines as human breast adenocarcinoma cell

line (MCF-7), gastric cancer cell line (MKN-45) and cervical cancer cell line (HeLa) using MTT assay [21]. Mouse normal fibroblasts (NIH-3T3) cell line was used as control. All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in DMEM medium containing 10% FBS, glutamine and pen/strep. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 and experiments were performed at 70-80% confluence at 75 cm cell culture flask. To determine the anticancer activity, cells were trypsinized with Trypsin-EDTA (0.25%) solution (Gibco, USA) and seeded into 96-well plates (1×10^4 cells/well). The cells were incubated new chalcone and pyrazoline derivatives at different concentrations for 24 h. After 24h, cells washed with PBS for elimination of derivatives and added to fresh 100 µL DMEM. Freshly prepared 10 µL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Vybrant, Invitrogen) used for viability testing. After adding MTT solution, plate was incubated for 4 h in a humidified atmosphere at 37°C incubator with 5% CO_2 in the air. At the end of the incubation, 100 µL of solubilization solution was added into each well. The color changes that occur after dissolving the formazan precipitate were measured using a microplate reader at 570 nm (Epoch, Biotek,USA). Cell viability was carried out in triplicate of each assay.

4.2.1. Caspase-3 activity assay

In order to determine the levels of the cell caspase-3 activity, cells were lysed with cell lysis buffer (50mM HEPES, pH 7.4, 0.1% CHAPS, 5mM dithiothreitol, 0.1mM ethylenediaminetetraacetic acid) and centrifuged for 10 min at 9000 rpm at 4°C after treatment of compounds for 24 h [21]. Using a commercial kit (Calbiochem 235419, San Diego, CA, USA) and following the manufacturer's instructions, the supernatant was used for measuring caspase-3 activity with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) substrate. Release of the pNA moiety was detected at 405 nm (εmM=10.5) by ELISA reader (Epoch, Biotek, USA) and concentration was calculated with pNA standards.

4.2.2. Western blot analysis for protein expression

Cells were treated with the final concentration of IC_{50} of synthesized compound **1f** and **2f** in 6 well plates and incubated for 24 h [22]. Treated cells were collected and lysed with RIPA lysis buffer (Thermo Scientific 89900, USA) included protease inhibitor cocktail (Sigma P2714, USA). Total cellular protein concentrations were measured by BCA protein assay (Thermo Scientific 23227, USA). Cellular proteins were denaturated with SDS-Laemmli buffer pH 6.8 (0.125 M Tris-HCl, 20% glycerol, 10% β-mercaptoethanol, 4% SDS, and 0.004% bromphenol

blue) and 25 µg protein was loaded to each well. SDS-PAGE (4–12%) gels were prepared and celular proteins resolved. Gels were transferred onto polyvinylidene fluoride membrane (PVDF) using transfer sytsem (Trans-Blot Turbo Transfer System, Biorad, USA) and blocked with 3% BSA (Capricorn Scientific, BSA-1T). After the blocking, the PVDF membranes were incubated with overnight 1:250 dilution specific primary antibodies (anti-Bax sc-7480, anti-Bcl2 sc-492, anti-GAPDH sc-25778, Santa Cruz Biotechnology, Heidelberg, Germany) at 4°C. Then, the PVDF membranes were washed with TBST (containing 0.1% Tween 20) and incubated with the 1:1000 dilution HRP-conjugated secondary antibodies antibody (anti-mouse; sc-2060 or anti-rabbit; sc-2004, Santa Cruz Biotechnology) for 2 h. After that, the PVDF membranes were incubated with ECL substrate (sc2048, Santa Cruz Biotechnology, Texas, USA) for the detection of the bands using imaging system (Syngene, Cambridge, UK). For the normalization of each band, GAPDH was used as a housekeeping protein as control. Finally, the expression levels of proteins were analyzed and quantified using Image J software (NIH, USA).

4.3. Physicochemical properties and pharmacokinetic profile predictions

All synthesized compounds were tested for their drug-like properties such as Lipinski rule of five and Veber rule. Their pharmacokinetic properties such as gastrointestinal absorption, brain permeation and P-glycoprotein substrate were predicted using Swissadme online server (http://www.swiss adme.ch/) for calculations.

4.4. Statistical Analysis

Student's t-test or analysis of variance (ANOVA) were used to analyze the differences among group means as appropriate. A p-value of 0.05 or less was considered as significant. All data is represented as standard deviation from the mean, unless otherwise indicated. All experiments were repeated in triplicate. Data from representative experiments are shown.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1. Half maximal inhibitory concentrations (IC_{50}) for the drugs under study at 24 h in HeLa, MCF-7, MKN-45 and NIH-3T3 cells.

IC-50 (μM) values				
Compound #	**HELA**	**MCF-7**	**MKN-45**	**NIH-3T3**
1a	45.61±2.62	>50	>50	>50
1b	>50	>50	>50	>50
1c	>50	>50	24.34±3.17	>50
1d	>50	10.72±1.68	>50	>50
1e	>50	>50	>50	>50
1f	26.66±2.73	9.41±2.19	5.17±3.54	>50
1g	>50	>50	>50	>50
1h	15.21±2.15	>50	>50	>50
1i	>50	24.06±4.58	0.04±0.02	22.01±3.71
1j	>50	>50	49.63±6.62	>50
1k	>50	0.66±0.17	0.07±0.03	0.79±0.21
1l	>50	17.34±1.41	27.90±3.46	3.13±1.11
1m	15.64±3.10	>50	>50	>50
1n	>50	>50	1.19±1.01	1.10±1.03
1o	>50	0.81±0.25	0.13±0.10	0.02±0.01
1p	>50	34.19±6.13	0.03±0.01	0.24±0.18
1r	>50	>50	1.21±0.64	>50
1s	>50	10.76±4.22	46.01±3.14	0.60±0.22
2a	>50	8.38±3.41	>50	1.26±1.09
2b	>50	2.30±1.18	>50	0.04±0.02
2c	>50	>50	>50	>50
2d	>50	7.17±1.19	>50	>50
2e	>50	>50	>50	>50
2f	17.96±3.34	0.69±0.13	0.88±0.16	>50
2g	>50	4.01±2.43	0.53±0.04	1.51±0.14
2h	5.14±1.66	>50	>50	>50
2i	>50	>50	0.50±0.22	>50
2j	>50	>50	>50	>50
2k	>50	>50	>50	>50
2l	>50	0.06±0.04	0.02±0.01	0.02±0.01
2m	3.33±1.02	>50	>50	>50
2n	>50	>50	>50	>50

2o	>50	>50	>50	>50
2p	>50	>50	>50	>50
2r	>50	>50	0.06±0.02	>50
2s	>50	0.16±0.04	0.02±0.01	0.05±0.02

Table 2. Lipinski and Veber Parameters of the synthesized compounds.

Code	Lipinski rule of five				Veber rule		Code	Lipinski rule of five				Veber rule	
	MW	**Log P**	***n*-ON**	**n-OHNH**	***n*-ROTB**	**TPSA**		**MW**	**Log P**	***n*-ON**	**n-OHNH**	***n*-ROTB**	**TPSA**
1a	345.37	4.29	3	1	6	46.17	**2a**	435.49	5.21	3	1	6	44.70
1b	363.36	4.66	4	1	6	46.17	**2b**	453.48	5.58	4	1	6	44.70
1c	379.81	4.77	3	1	6	46.17	**2c**	469.94	5.68	3	1	6	44.70
1d	390.36	3.21	5	1	7	91.99	**2d**	480.49	4.19	5	1	7	90.52
1e	359.39	4.50	3	1	6	46.17	**2e**	449.52	5.41	3	1	6	44.70
1f	335.33	2.64	4	1	6	59.31	**2f**	425.45	4.02	4	1	6	57.84
1g	372.37	2.83	4	1	7	91.99	**2g**	462.50	3.82	4	1	7	90.52
1h	390.36	3.21	5	1	7	91.99	**2h**	480.49	4.19	5	1	7	90.52
1i	406.82	3.32	4	1	7	91.99	**2i**	496.94	4.29	4	1	7	90.52
1j	417.37	1.89	6	1	8	137.81	**2j**	507.50	2.91	6	1	8	136.34
1k	386.40	3.05	4	1	7	91.99	**2k**	476.53	4.02	4	1	7	90.52
1l	362.34	1.24	5	1	7	105.13	**2l**	452.46	2.67	5	1	7	103.66
1m	341.40	4.13	2	1	6	46.17	**2m**	431.53	5.04	2	1	6	44.70
1n	359.39	4.50	3	1	6	46.17	**2n**	449.52	5.41	3	1	6	44.70
1o	375.85	4.61	2	1	6	46.17	**2o**	465.97	5.50	2	1	6	44.70
1p	386.40	3.05	4	1	7	91.99	**2p**	476.53	4.02	4	1	7	90.52
1r	355.43	4.34	2	1	6	46.17	**2r**	445.55	5.24	2	1	6	44.70
1s	331.36	2.49	3	1	6	59.31	**2s**	421.49	3.85	3	1	6	57.84

Table 3. Drug-like properties of compound **1f** and **2f**.

Drug-likeness parameters	**Compound 1f**	**Compound 2f**
Log S	-4.38 (Moderately soluble)	-5.80 (Moderately soluble)
Egan logP<5.88 TPSA<131.6	Yes	Yes
Ghose 160<MW<480 -0.4<logP<5.6 40<MR<130 20<atoms<30	Yes	Yes
Bioavailability score	0.55	0.55

MR: Molar refractivity

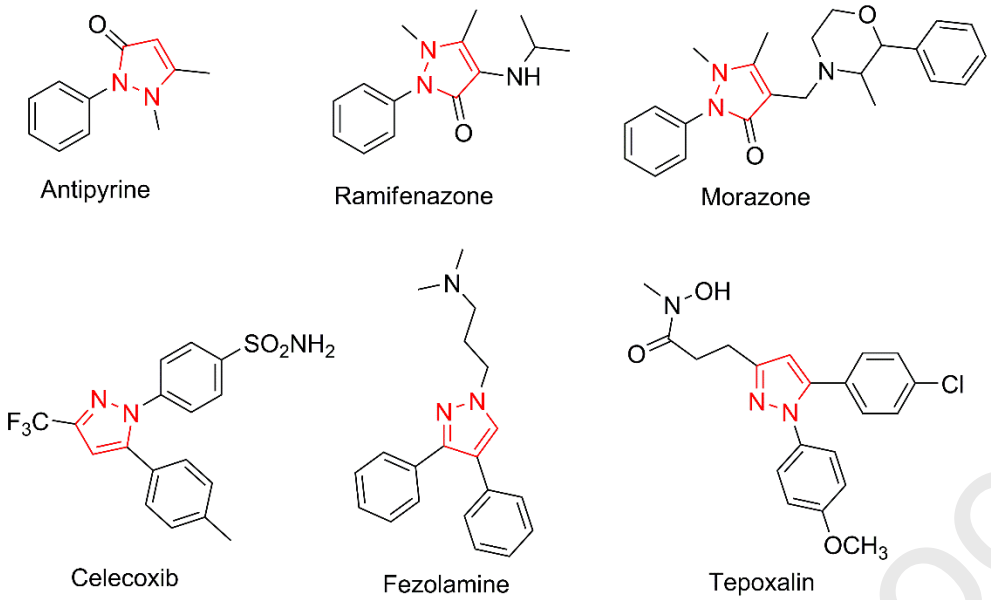

Figure 1: Chemical structures of pyrazole and pyrazoline derivatives

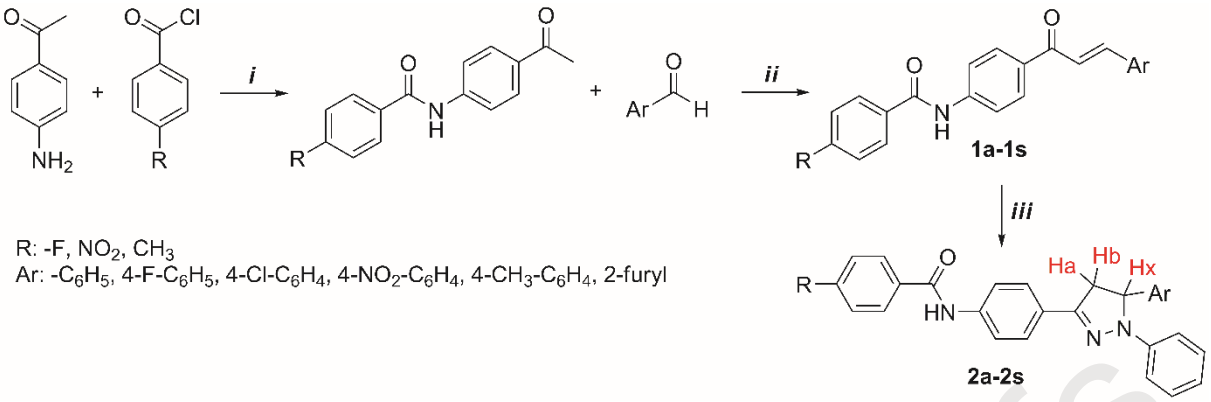

Figure 2: The synthetic pathway of compounds. Reagents and conditions: (i) Chloroform, reflux, 8 h; (ii) Ethanol, KOH, 10 h; (iii) Phenylhydrazine hydrochloride, acetic acid, reflux, 12 h.

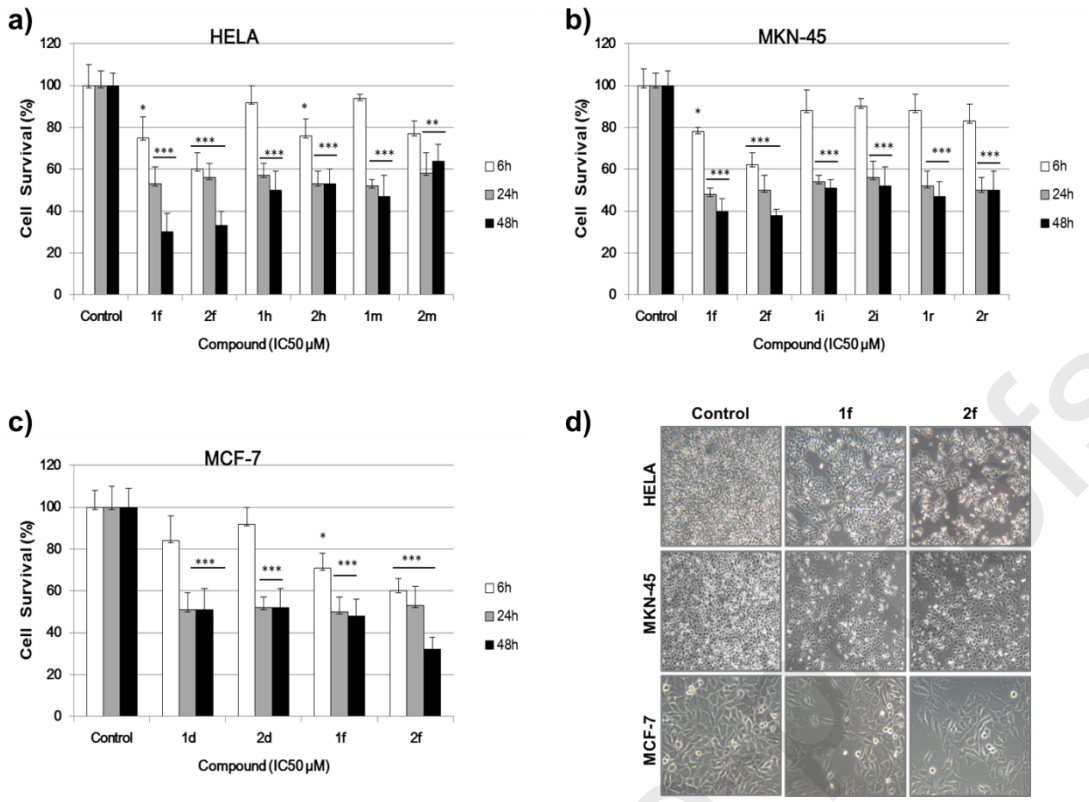

Figure 3. Effect of compounds on cell survival in human HELA, MKN-45 and MCF-7 cancer cells. **a)** Effect of compound **1f, 2f, 1h, 2h, 1m** and **2m** (IC_{50} µM) on cell survival of HELA cells for 6h, 24 h and 48h. **b)** Effect of compound **1f, 2f, 1i, 2i, 1r** and **2r** (IC_{50} µM) on cell survival of MKN-45 cells for 6h, 24 h and 48h. **c)** Effect of compound **1d, 2d, 1f** and **2f** (IC_{50} µM) on cell survival of MCF-7 cells for 6h, 24 h and 48h. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control of each cells MCF-7 cells). **d)** Morphological changes in response to compounds. HELA, MKN-45 and MCF-7 cells were treated with the **1f** and **2f** compound at different IC_{50} concentations (µM) for 24 h. (All experiments were repeated in triplicate)

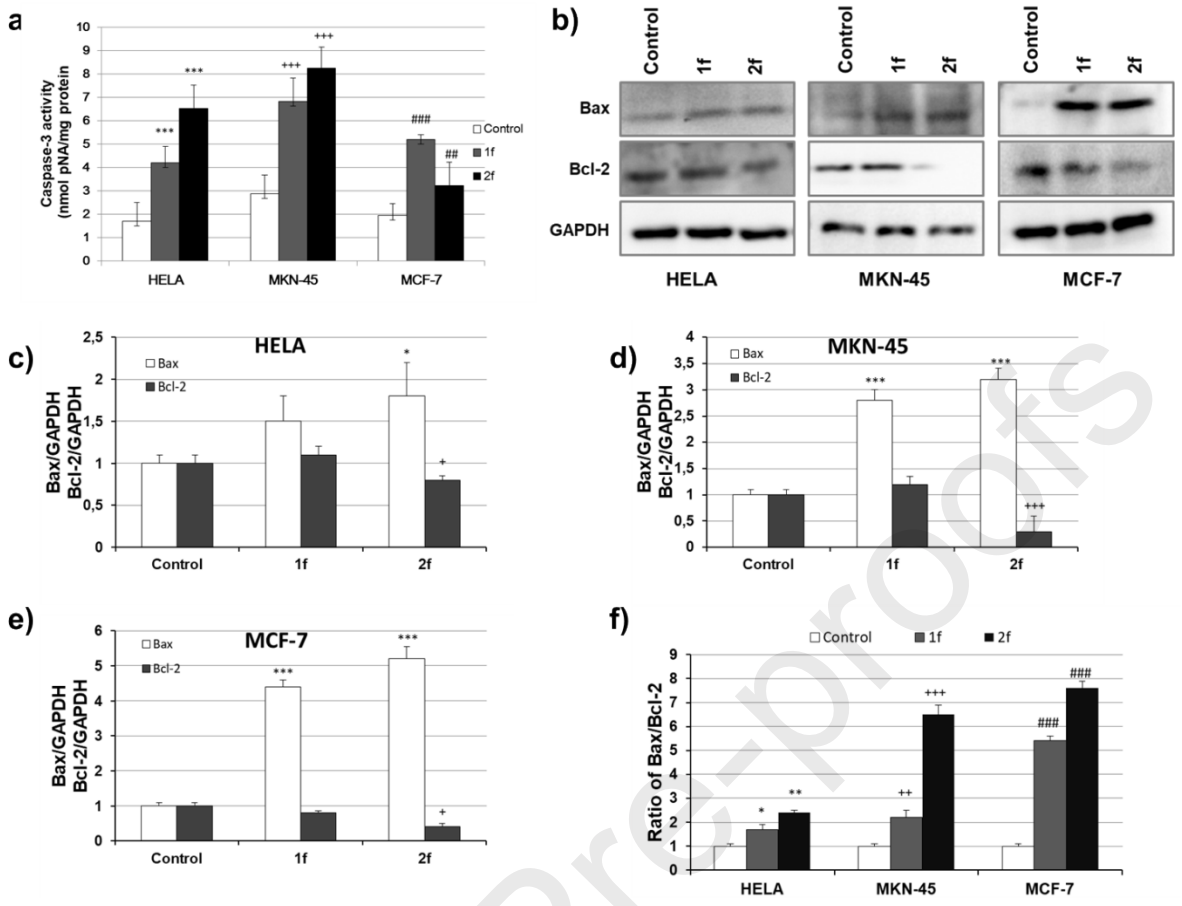

Figure 4. Effects of compound **1f** and **2f** on apoptosis in HELA, MKN-45 and MCF-7 cancer cells. **a)** Cells were treated with compound **1f** and **2f** the at a final concentration of IC_{50} µM and assessed for caspase 3 activity (***$p < 0.001$ compared to control in HELA cells, +++$p < 0.001$ compared to control in MKN-45 cells, ##$p < 0.01$. ###$p < 0.001$ compared to control in MCF-7 cells). **b)** Effects of compound **1f** and **2f** treatment at 24 h on the Bcl-2 and Bax proteins in HELA, MKN-45 and MCF-7 cancer cells, representatively. **c-e)** Western blot densitometry analysis of expression levels of Bcl-2 and Bax proteins. **f)** Ratio of Bax/Bcl-2 expression in HELA, MKN-45 and MCF-7 cancer cells (*$p < 0.05$, **$p < 0.01$ compared to control in HELA cells, ++$p < 0.01$, +++$p < 0.001$ compared to control in MKN-45 cells, ###$p < 0.001$ compared to control in MCF-7 cells). Each protein band was normalized to the intensity of GAPDH used. (All experiments were repeated in triplicate)

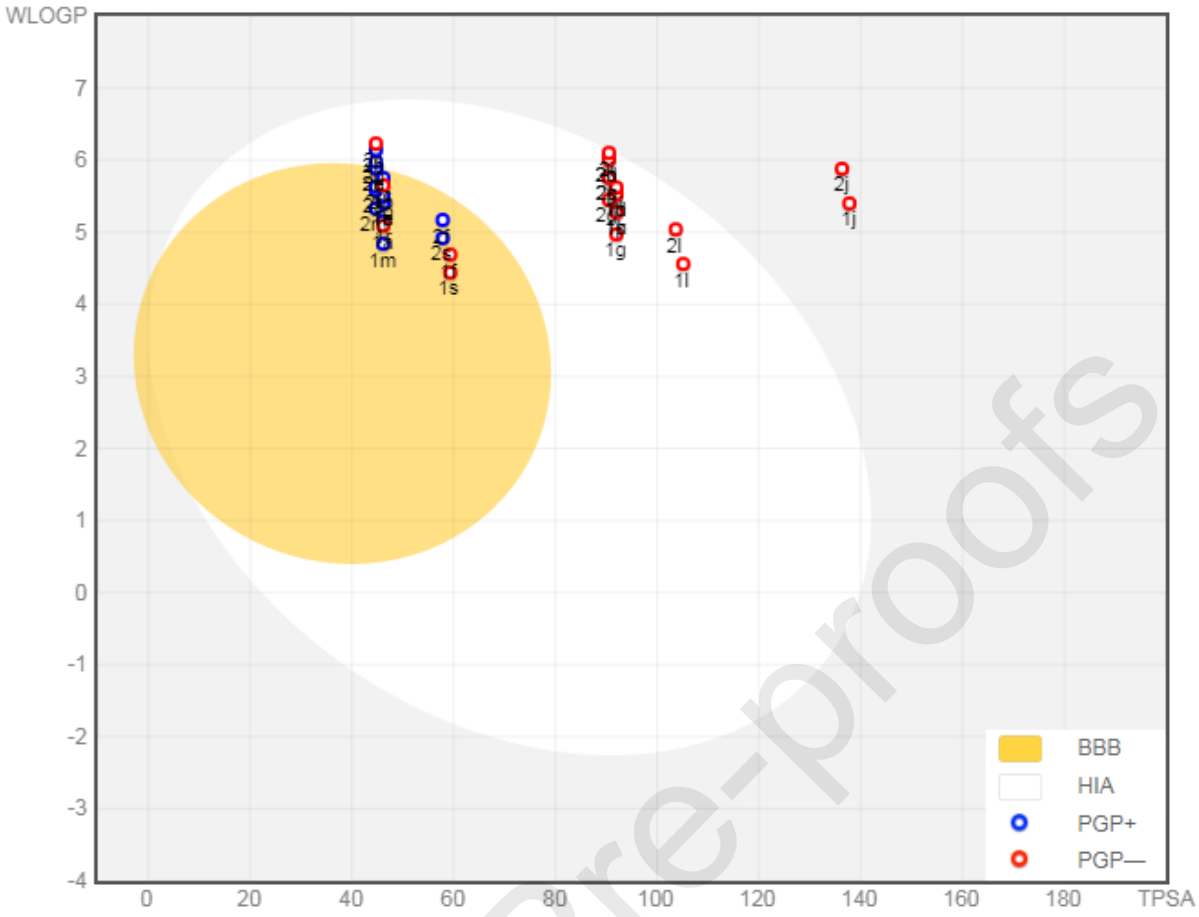

Figure 5. The boiled egg plot for prediction of the pharmacokinetics of synthesized compounds.