

## SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL PYRAZOLO[3,4-D]PYRIMIDINE DERIVATIVES OF EXPECTED ANTICANCER ACTIVITY

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### Abstract

A series of novel pyrazolo[3,4-*d*]pyrimidine derivatives have been designed based on chemical modifications on the general features of the reported and clinically used EGFR-TKIs such as replacing of quinazoline moiety of reported EGFR-TKIs as erlotinib by pyrazolo[3,4-*d*]pyrimidine nucleus, introducing different hydrophobic moieties including phenyl, aromatic heterocyclic, fused aromatic or aliphatic structures, introducing different linkers which may be one atom, two atoms, three atoms, four atoms and five atoms and introducing of phenyl ring at position-3 of 1H-pyrazolo[3,4-*d*]pyrimidine nucleus to occupy the hydrophobic region II of ATP binding site. All the new synthesized compounds were biologically screened *in vitro* for their cytotoxic activities against four cancer cell lines namely, HepG-2, MCF-7, HCT-116, and Hela. The results of cytotoxic evaluation indicated that compound VI was found to be the most prominent broad-spectrum cytotoxic activity and significantly more potent than doxorubicin with IC<sub>50</sub> values of 6.18, 6.48, 4.03, and 5.82 μM against tested cell lines. In addition, compounds IXa,b displayed promising cytotoxic effect against all tested cell lines with IC<sub>50</sub> values less than 30 μM compared with doxorubicin as a control drug. Besides, compound X possessed excellent anti-proliferative activities against the four cell lines with IC<sub>50</sub> values ranging from 18 μM to 39.5 μM. Structural pharmacophoric features indicated that pyrazolo[3,4-*d*]pyrimidine scaffold having a four atoms linker as thiosemicarbazide moiety as compounds IXa,b which substituted with aliphatic moiety at the 4-position was more potent than those possessing one atom, two atoms, three atoms and five atoms linkers which lead to significant decrease in cytotoxic

**Keywords:** EGFR, Pyrazolo[3,4-*d*]pyrimidines; Anticancer activity

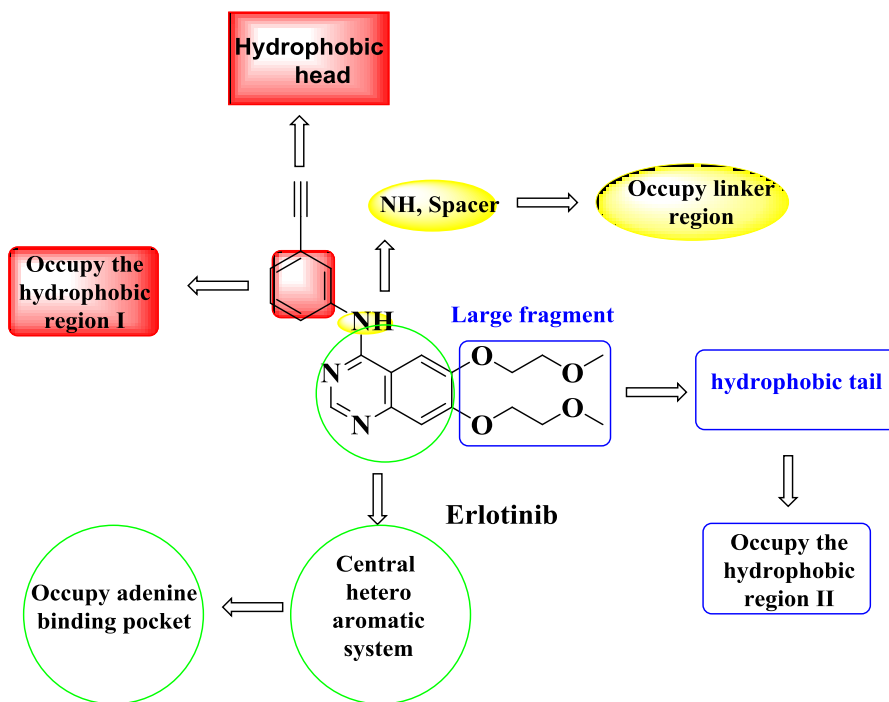
## 1- Introduction

cancer is considered as the second cause of death in the world with every 1 in 6 deaths were attributed to cancer(Society, 2016). Since the chemotherapy remains one of the most active and common routes for cancer therapy but is also related with severe toxicity and poor tolerance(Horiuchi *et al.*, 2012). Thus, there has been a persistence ever-increasing effort by the pharmaceutical industries and the researchers to identify and develop new cancer chemotherapeutics for an effective treatment with reduced side effects. (Cohen, 2002). The chemistry of pyrazolo[3,4-d]pyrimidine derivatives has win great attention due to their structural similarity with purines and so several pyrazolo[3,4-d]pyrimidine derivatives exhibited good anticancer activity(Cheng and Robins, 1956) (El-Enany *et al.*, 2010) (El Hamid *et al.*, 2012) (Kandeel *et al.*, 2012) by acting as ATP competitive inhibitor for several kinase enzymes. Indeed, many pyrazolo[3,4-d]pyrimidines were reported to give promising anti-tumor activity(Rashad *et al.*, 2011) (Ghorab *et al.*, 2010) (El-Enany *et al.*, 2010). Their cytotoxic activities may be due to inhibition of several enzymes such as epidermal growth factor receptor (EGFR) inhibitors(Schenone, Bruno, Bondavalli, Ranise, Mosti, Menozzi, Fossa, Manetti, *et al.*, 2004) tyrosine kinase(Schenone, Bruno, Bondavalli, Ranise, Mosti, Menozzi, Fossa, Donnini, *et al.*, 2004) (Ducray *et al.*, 2008), cyclin dependent kinase (CDK) (Kim *et al.*, 2003) (Markwalder *et al.*, 2004), Src kinase(Kumar *et al.*, 2011) and glycogen Synthase kinase (GSK) (Peat *et al.*, 2004).

## 2. Rational Drug Design

In this work, we have carried out the chemical modifications on the general features of the reported and clinically used EGFR-TKIs as erlotinib 1(Sharma *et al.*, 2016) (Mowafy *et al.*, 2016). The modification comprises a replacement of the quinazoline moiety of erlotinib by 1H-pyrazolo[3,4-d]pyrimidine nucleus in an attempt to enhance the cytotoxic activity. The second modification was done by introducing different hydrophobic moieties including phenyl, aromatic heterocyclic, fused aromatic or aliphatic structures.

The third modification was done by introducing different linkers may be one atom (e.g. imino group as compound XI), two atoms (e.g. hydrazono group as compound XII), three atoms (e.g. ketohydrazinyl group as compounds X), four atoms (e.g. thiosemicarbazide moiety as compounds IX), five atoms e.g. cyclic structure (e.g. pyrrole and pyrazole ring as compound VII and VIII). The fourth modification was done introducing of phenyl ring at position-3 of 1H-pyrazolo[3,4-d]pyrimidine nucleus to occupy the hydrophobic region II of ATP binding site.



(Figure 1). The basic structural pharmacophoric requirements for erlotinib as reported EGFR-TK inhibitor.

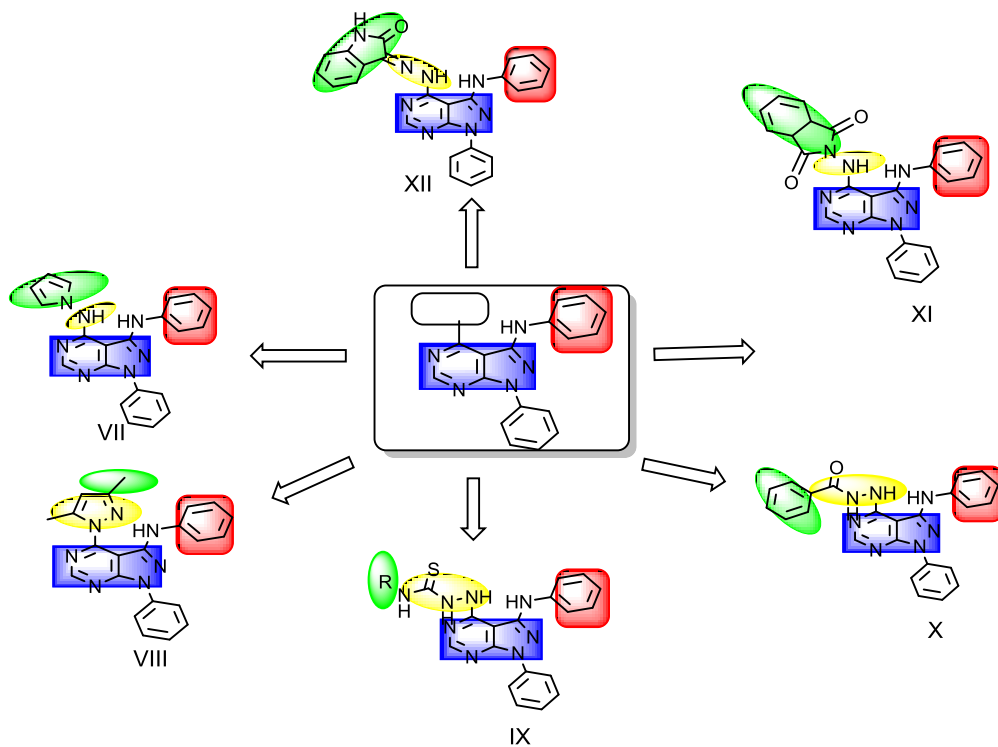
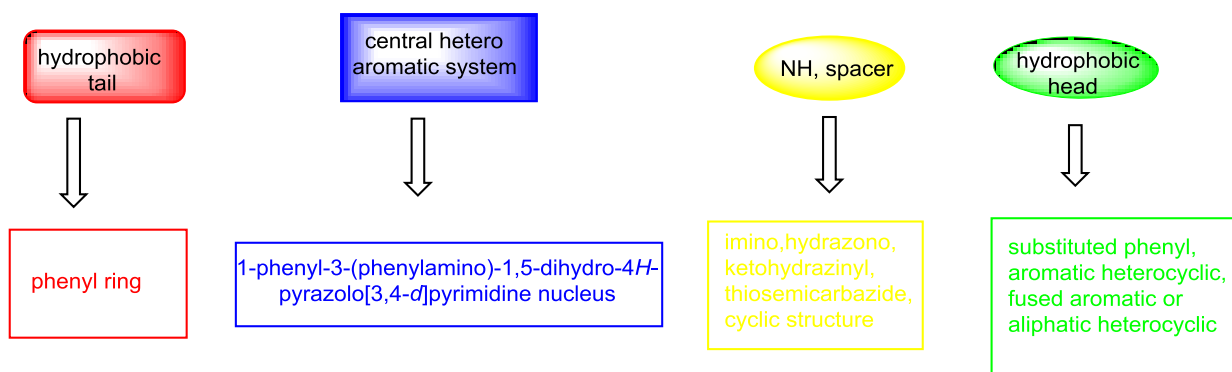


Fig. 2. Rational of molecular design of the new proposed EGFR-TK inhibitors.



**Fig. 2. Summary for the possible modifications of EGFR-TK inhibitors.**

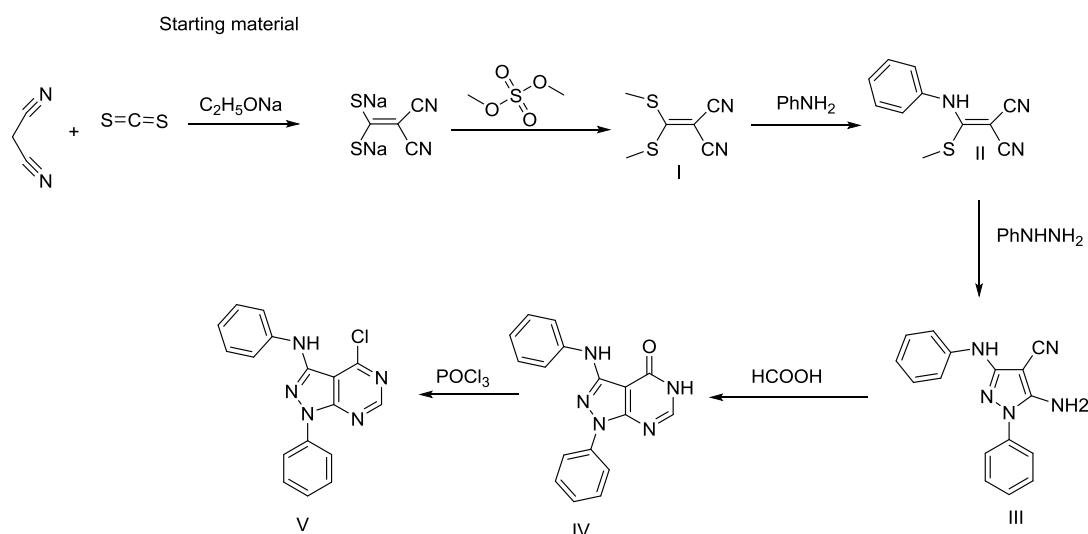
### 3- Results and Discussion

#### 3.1 Chemistry

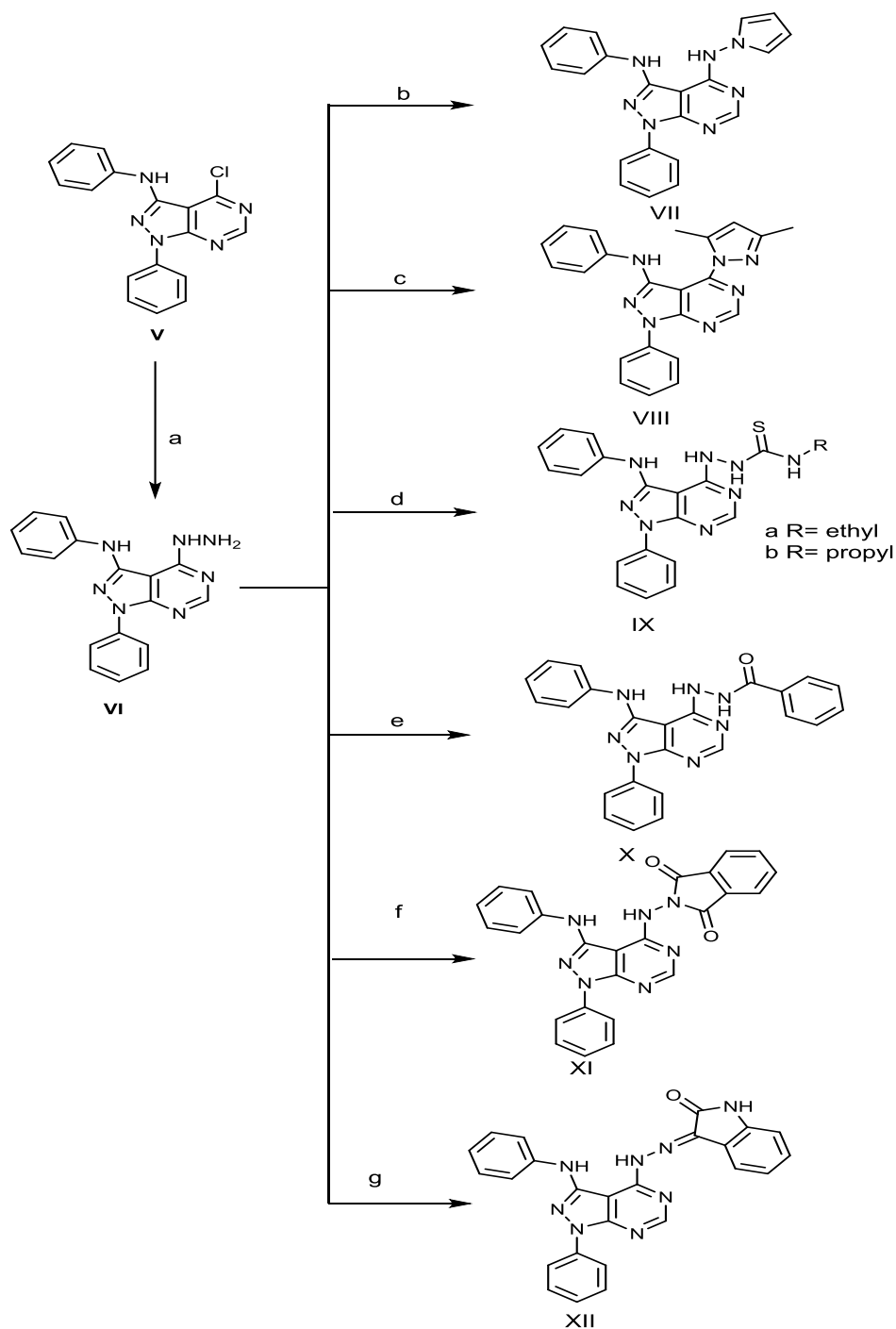
The designed compounds were synthesized as outlined in Schemes 1 & 2. 2-(bis(methylthio)-methylene)malononitrile I (Traxler *et al.*, 1997a) was allowed to react with aniline to give 2-((methylthio)(phenylamino)methylene)malononitrile II (Li *et al.*, 2014) compound II was reacted phenyl hydrazine to produce 5-Amino-3-(methylthio)-1-phenyl-1H-pyrazolo-4-carbonitrile III (Traxler *et al.*, 1997b) The IR spectrum of III demonstrated stretching bands at 3353, 3309, 3209 and 2206  $\text{cm}^{-1}$  corresponding to  $\text{NH}_2$ , NH and CN respectively. Moreover,  $^1\text{H}$  NMR of this compound showed two exchangeable signals at  $\delta$  4.73 and 9.89 ppm corresponding to  $\text{NH}_2$  and NH, respectively and an increase in the integration of the aromatic protons indicating the presence of additional aromatic ring. The mass spectrum of compound III showed a molecular ion peak at  $m/z$  275 which represents the base peak. Compound III reacted with formic acid to give 1-phenyl-3-(phenylamino)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one IV The IR spectrum of IV demonstrated stretching bands at 3356  $\text{cm}^{-1}$  corresponding to NH. Moreover,  $^1\text{H}$  NMR of this compound showed two signals at  $\delta$  12.48 and 8.19 ppm corresponding to NH and CH pyrimine respectively.  $^{13}\text{C}$  NMR spectrum of IV supported the carbon skeleton of the obtained structure and revealed the presence of an additional C=O signals at 158.4. The mass spectrum of compound IV showed a molecular ion peak at  $m/z$  303 which represents the base peak. Compound IV converted to compound V by phosphorousoxychloride The IR spectrum of V showing absence of C=O stretching band. The mas spectrum of compound V showed peaks at  $m/z$  323 and 321 correspond to  $\text{M}^+$  and  $\text{M}+2$  respectively at the ratio 3:1 which confirm the presence of chlorine atom, and molecular ion peak at  $m/z$  321 represents the base peak.

Refluxing compound V with hydrazine hydrate afforded the target compound VI the IR spectrum of VI demonstrated stretching bands at 3444, 3352 and 3190  $\text{cm}^{-1}$  corresponding to  $\text{NH}_2$  and NH, respectively. Moreover,  $^1\text{H}$  NMR of this compound showed two exchangeable signals at  $\delta$  4.73 and 9.89 ppm corresponding to  $\text{NH}_2$  and NH, respectively. When compound VI react with 2,5-diethoxytetrahydrofuran give

compound VII  $^1\text{H}$  NMR of this compound showed two signals at  $\delta$  6.93 and 6.84 ppm corresponding to H2,H5 and H3,H4 of pyrrole respectively. Treatment of compound VI with acetylacetone yielded a pyrazolo derivative VIII (cyclized product). Compound VIII was characterized by  $^1\text{H}$  NMR spectrum with presence of aliphatic protons. The IR spectrum of this compound demonstrated presence of stretching band of aliphatic group due to the cyclization of the final compound. When compound VI reacted with ethyl or propyl isothiocyanate afforded compounds IXa-b respectively  $^1\text{H}$  NMR of compound IXa showed two signals at  $\delta$  3.58 and 1.19 ppm corresponding to two aliphatic groups while compound IXb showed three signals at  $\delta$  3.85, 1.62 and 1.22 ppm corresponding to three aliphatic groups. On the other hand, compound VI was allowed to react with benzoylchloride in presence of catalytic amount of TEA to produce the target benzohydrazide derivatives X.  $^1\text{H}$  NMR spectra of compounds X exhibited three  $\text{D}_2\text{O}$  exchangeable signals corresponding to three NH groups. The IR spectra of this compound demonstrated stretching band  $1688\text{ cm}^{-1}$  corresponding to  $\text{C}=\text{O}$  groups. Additionally, phthalimid derivative XI was prepared by the reaction of compound VI with commercially available phthalic anhydride the produced compound showed a characteristic  $\text{D}_2\text{O}$  exchangeable signal corresponding to the additional NH group. The IR spectrum of this compound demonstrated stretching band at  $1715\text{--}1730\text{ cm}^{-1}$  corresponding to the two  $\text{C}=\text{O}$  groups. Finally, treatment of compound VI with commercially available isatin in the presence of glacial acetic acid afforded the target compound XII.



Scheme 1. Reagents and conditions of reaction; (a) aniline, absolute ethanol, reflux, 6 h; (b) phenyl hydrazine, absolute ethanol, reflux, 12 h; (c) formic acid reflux, 14 h; (d) phosphoryl trichloride, reflux, 6h;



Scheme 2. Reagents and conditions of reaction; (a) hydrazine hydrate 99%, reflux, 8h; (b) 2,5-diethoxytetrahydrofuran, glacial acetic acid, reflux 16h; (c) acetyl acetone, glacial acetic acid, reflux 48 h.(d) app. isothiocyanates, butanol, RT; (e) benzoyl chlorides, DMF, RT; (f) phthalic anhydride, glacial acetic acid, reflux 16h; (g) isatin, glacial acetic acid, reflux, 24h.

### 3.2 Biological Evaluation

#### 3.2.1 *In Vitro* Anticancer Screening

According to the rational drug design, a series of novel pyrazolopyrimidine derivatives were designed and synthesized. Consequently, the new synthesized compounds were evaluated for their *in vitro* cytotoxic activity against four different cancer cell lines namely, hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7), colorectal carcinoma (HCT-116) and cervical cancer (Hela) via standard MTT method (Mosmann, 1983) (Denizot and Lang, 1986) (Thabrew *et al.*, 1997),

From the obtained anticancer results, it is evident that the screened compounds displayed different levels of cytotoxicity ranging from potent, moderate, weak, and inactive cytotoxicity against all tested cell lines. Therefore, data represented in (Table 1) revealed that, compound VI was found to be significantly more potent and efficient than doxorubicin with IC<sub>50</sub> values of 6.18, 6.48, 4.03, and 5.82 μM against tested cell lines. Moreover, compounds IXa,b was found to be the most potent derivatives against the four cell lines with IC<sub>50</sub> values less than 30 μM compared with an anticancer drug, doxorubicin as control. Besides, compounds X possessed moderate anti-proliferative activities against the four cell lines with IC<sub>50</sub> values ranging from 18 μM to 40 μM. Furthermore, several derivative compounds such as IV, V, XI and XII showed weak anti-proliferative activities with IC<sub>50</sub> values ranging from 29 to 63 μM. Finally, compounds III, VII and VIII appeared to be inactive against tested cell lines.

Compounds	In vitro Cytotoxicity IC <sub>50</sub> (μM)•			
	HePG2	Hela	HCT-116	MCF-7
SOR	9.18±0.6	8.16±0.6	5.47±0.3	7.26±0.3
DOX	4.50±0.2	5.57±0.4	5.23±0.3	4.17±0.2
III	>100	91.74±5.3	>100	80.60±4.2
IV	60.18±4.2	72.39±4.2	37.89±2.8	59.34±3.6
V	52.81±4.1	66.32±3.2	72.59±4.1	64.08±3.5
VI	6.18±0.5	5.82±0.3	4.03±0.2	6.48±0.4
VII	82.92±4.7	>100	96.81±4.9	90.08±4.5
VIII	99.43±4.9	94.11±5.3	>100	76.14±4.4
IXa	19.35±1.6	34.41±3.6	17.66±1.3	30.16±3.1
IXb	15.32±3.5	25.62±3.6	14.76±3.2	28.17±3.5
X	28.43±4.0	18.11±1.9	39.50±3.3	36.94±3.9
XI	64.14±4.2	42.12±4.6	43.97±4.1	42.71±4.7
XII	53.38±4.3	28.65±2.6	31.03±3.0	32.56±3.7

Table 1 *In- vitro* anti-proliferative activities towards HePG2, Hela, HCT-116 and MCF-7 cell lines.

• IC<sub>50</sub> (μM) : 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak)

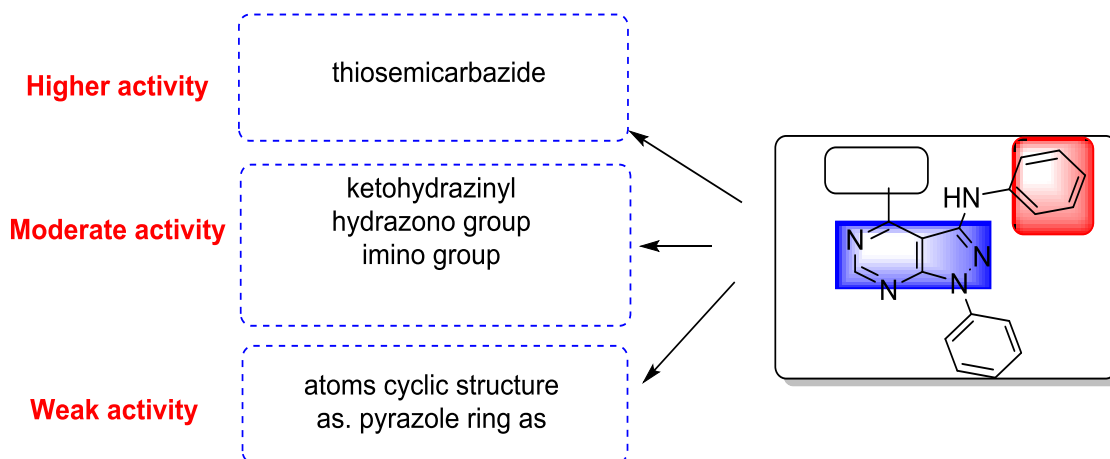
and above 100 (non-cytotoxic)

•• SOR : Sorafenib

••• DOX : Doxorubicin

### 3.2.2 Structure-activity relationship (SAR)

Generally the study of the structure–activity relationship (SAR) based on the cytotoxicity results indicated that the activity was extremely influenced by the nature of various substituents present at C-4 (aromatic ring) on pyrazolo[3,4-d]pyrimidine scaffold. It was found that the activity decreased in the order four atoms thiosemicarbazide (compounds IXa-b) > three atoms ketohydrazinyl group (compounds X) > two atoms hydrazono group (compound XII) > one atom imino group (compound XI) > five atoms cyclic structure e.g. pyrazole ring as (compounds VII and VIII).



**Fig. 3:** SAR of the synthesized compounds

## 4. Experimental

### 4.1. Chemistry

#### 4.1.1. General

Melting points were measured on a Gallen-kamp melting point apparatus and were uncorrected. The IR spectra were recorded on Nicolet IR 200 FT IR spectrophotometer using KBr discs ( $\lambda$  max in  $\text{cm}^{-1}$ ).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were performed on Gemini 300BB spectrometer at 300MHz and Bruker spectrometer at 75MHz, respectively. TMS was used as internal standard and  $\text{DMSO-}d_6$  as solvent. The chemical shifts were reported in ppm ( $\delta$ ) and coupling constant (J) values were given in Hertz (Hz). Signal multiplicities were represented by s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). All of the new compounds were analyzed for C, H and N and agreed with the proposed structures within  $\pm 0.4\%$  of the theoretical values by the automated CHN analyzer. Mass spectra were recorded on a unit of Shimadzu GCMS-QP/MS-QP5050A spectrometer operating at 70 eV. The purity of the compounds was checked by thin layer chromatography (TLC) using Merck silica gel 60 F254 precoated sheets.



**5-Amino-1-phenyl-3-(phenylamino)-1H-pyrazole-4-carbonitrile (III)**

A solution of 2-((Methylthio)(phenylamino)methylene)malononitrile ( 2.15 g, 0.01 mol) and phenylhydrazine ( 1.08 g, 1 mL, 0.01 mol) in absolute ethanol (20 mL) was heated under reflux for 3 h. The reaction mixture was poured in to water (200 mL). The yellow precipitate formed was filtered, dried and crystallized from methanol

Yellow needle, yield: 90 %; m.p. 165 °C. IR (KBr)  $\text{cm}^{-1}$ : 3356, 3309 (NH<sub>2</sub>), 3209 (NH), 3055 (CH aromatic), 2206 (CN). <sup>1</sup>HNMR (DMSO-*d*<sub>6</sub>)  $\delta$  ppm: 8.65 (s, 1H, NH), 7.59-7.46.13 (m, 4H, Ar-H), 7.23-7.21 (m, 2H, Ar-H), 6.82-6.80 (m, 2H, Ar-H), 6.82-6.80 (m, 2H, Ar-H), 5.74(s, 2H, NH<sub>2</sub>). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  (ppm): 61.89, 120.96, 121.04, 126.26, 129.26, 129.56, 139.00, 141.42, 147.92, 150.07, and 151.50. (Aromatic carbons). 117.46 (CN). MS (m/z): 275 (C<sub>16</sub>H<sub>13</sub>N<sub>5</sub>, 100%, M<sup>+</sup>), 259 (C<sub>16</sub>H<sub>11</sub>N<sub>4</sub>, 1.51%), 183 (C<sub>10</sub>H<sub>7</sub>N<sub>4</sub>, 1.47%). Anal. Calc. for: (C<sub>16</sub>H<sub>13</sub>N<sub>5</sub>) (M.W. = 275): C, 69.80; H, 4.76; N, 25.44%; Found: C, 69.89; H, 4.73; N, 25.47%.

**1-Phenyl-3-(phenylamino)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (IV)**

A mixture of pyrazole derivative 7 (2.75 g, 0.01mol) and formic acid (85%, 40 mL) was heated under reflux for 8 h. The reaction was cooled, and the separated solid was filtered, dried and crystallized from formic acid

White solid, Yield: 85%; m.p. 230 °C. IR (KBr)  $\text{cm}^{-1}$ : 3356 (NH), 3024 (CH aromatic), 1674 (C=O). <sup>1</sup>HNMR (DMSO-*d*<sub>6</sub>)  $\delta$  ppm: 12.48 (s, 1H, NH), 8.19 (s, 1H, pyrimidine-H2), 8.13 (s, 1H, NH), 8.11 (d, 2H, *J* = 6.64 Hz, Phenyl-H2,H6), 7.81 (d, 2H, *J* = 7.96 Hz, NHPhenyl-H2,H6), 7.57 (t, 2H, *J* = 7.56 Hz, Phenyl-H3,H5), 7.34-7.31 (m, 3H, *J* = 7.48 Hz, Ar-H), 6.95 (t, 1H, *J* = 7.16 Hz, NHPhenyl-H4). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  (ppm): 97.09, 117.44, 121.02, 121.08, 126.34, 129.29, 129.59, 138.94, 141.39, 147.90, 150.01 and 151.49 (Aromatic carbons), 158.48 (C=O). MS (m/z): 303 (C<sub>17</sub>H<sub>13</sub>N<sub>5</sub>O, 100%, M<sup>+</sup>), 287 (C<sub>16</sub>H<sub>11</sub>N<sub>5</sub>, 1.97%). Anal. Calc. for: (C<sub>17</sub>H<sub>13</sub>N<sub>5</sub>O) (M.W. = 303): C, 67.32; H, 4.32; N, 23.09%; Found: C, 67.28; H, 4.36; N, 23.12%.

**4-Chloro-N,1-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-3-amine (V)**

A suspension of **IV** (3.03g, 0.01mol) and phosphorous oxychloride (40 mL) was heated under refluxed for 3 h. The solution was cooled and then poured onto ice water. The precipitated product was filtered, dried, and crystallized from ethanol.

White solid, Yield: 85%; m.p. 130 °C. IR (KBr)  $\text{cm}^{-1}$ : 3417 (NH), 3005 (CH aromatic). <sup>1</sup>HNMR (DMSO-*d*<sub>6</sub>)  $\delta$  ppm: 8.86 (s, 1H, pyrimidine-H2), 8.39 (s, 1H, NH), 8.20 (d, 2H, *J* = 7.88 Hz, Phenyl-H2,H6), 7.79 (d, 2H, *J* = 7.88 Hz, NHPhenyl-H2,H6), 7.60 (t, 2H, *J* = 7.72 Hz, Phenyl-H3,H5), 7.39-7.33 (m, 3H, aromatic ), 7.02 (t, 1H, *J* = 7.32 Hz, NHPhenyl-H4). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  (ppm): 107.12, 118.33, 120.77, 121.94, 126.58, 129.31, 129.74, 138.46, 141.22, 144.27, 152.47, 153.69 and 156.51. (Aromatic carbons). MS (m/z): 321 (C<sub>17</sub>H<sub>12</sub>ClN<sub>5</sub>, 100%, M<sup>+</sup>), 323 (C<sub>17</sub>H<sub>12</sub>ClN<sub>5</sub>, 34.17%, M+2). Anal. Calc. for: (C<sub>17</sub>H<sub>12</sub>ClN<sub>5</sub>) (M.W. = 321): C, 63.46; H, 3.76; N, 21.77%; Found: C, 63.39; H, 3.90; N, 21.59%.

**4-Hydrazinyl-N,1-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-3-amine (VI)**

To a solution of V (3.89 g, 0.01 mol) in absolute ethanol (50 mL), hydrazine-hydrate (1 mL, 0.02 mol) was added and the reaction mixture was heated under reflux the solid formed collected, separated and crystallized from ethanol.

White solid, Yield: 70%; m.p. 160 °C. IR (KBr)  $\text{cm}^{-1}$ : 3294 (NH), 3055 (CH aromatic), 2993 (CH aliphatic). MS (m/z): 317 ( $\text{C}_{17}\text{H}_{15}\text{N}_7$ , 19.81%,  $\text{M}^+$ ), 301 ( $\text{C}_{17}\text{H}_{13}\text{N}_6$ , 25.97), 286 ( $\text{C}_{17}\text{H}_{12}\text{N}_5$ , 2.87). Anal. Calc. for: ( $\text{C}_{17}\text{H}_{15}\text{N}_7$  M.W. = 317): C, 64.34; H, 4.76; N, 30.90%; Found: C, 64.15; H, 4.69; N, 30.85%.

**N3,1-diphenyl-N4-(1H-pyrrol-1-yl)-1H-pyrazolo[3,4-d]pyrimidine-3,4-diamine(VII)**

To a solution of VI (0.001mol) in glacial acetic acid (20mL), 2,5-diethoxytetrahydrofuran (0.001mol) was added. The mixture was heated under reflux for 16h. After completion of the reaction mixture, it was concentrated and allowed to cool. The separated solid was filtered and crystallized from methanol to produce the target compound VII

White solid, Yield: 85%; m.p. 240 °C. IR (KBr)  $\text{cm}^{-1}$ : 3322 (NH), 3014 (CH aromatic).  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 8.94 (s, 1H, NH), 8.26 (s, 1H, pyrimidine-H2), 8.14 (d, 2H,  $J = 6.64$  Hz, Phenyl-H2,H6), 7.84 (d, 2H,  $J = 7.96$  Hz, NHPhenyl-H2,H6), 7.59 (t, 2H,  $J = 7.56$  Hz, Phenyl-H3,H5), 7.32-7.16 (m, 4H,  $J = 7.48$  Hz, aromatic), 6.83 (d, 2H,  $J = 7.16$  Hz, pyrrole- H2H5), 6.04 (d, 2H,  $J = 7.16$  Hz, pyrrole- H3H4), 3.95 (s, 1H, NH). MS (m/z): 367 ( $\text{C}_{21}\text{H}_{17}\text{N}_7$ , 100%,  $\text{M}^+$ ). Anal. Calc. for: ( $\text{C}_{21}\text{H}_{17}\text{N}_7$ ) (M.W. = 367): C, 68.65; H, 4.66; N, 26.69%; Found: C, 68.82; H, 4.79; N, 26.91%.

**N4-(3,5-Dimethyl-1H-pyrazol-1-yl)-N3,1-diphenyl-1H-pyrazolo[3,4-d]pyrimidine-3,4-diamine(VIII)**

A mixture of compound VI (0.3, 0.001mol) and acetyl acetone (0.1g, 0.01mol) in glacial acetic acid (20mL) was heated under reflux for 48h. Then, the reaction mixture was poured onto crushed ice with continuous stirring and filtered. The obtained solid was crystallized from ethanol to yield the targeted compound VIII.

White solid, Yield: 75%; m.p. 245 °C. IR (KBr)  $\text{cm}^{-1}$ : 3333 (NH), 3024 (CH aromatic), 2985 (CH aliphatic).  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 8.54 (s, 1H, pyrimidine-H2), 8.16 (s, 1H, NH), 8.04 (d, 2H,  $J = 6.64$  Hz, Phenyl-H2,H6), 7.64 (d, 2H,  $J = 7.96$  Hz, NHPhenyl-H2,H6), 7.39 (t, 2H,  $J = 7.56$  Hz, Phenyl-H3,H5), 7.19 (t, 1H,  $J = 7.66$  Hz, Phenyl- H4), 7.12-7.06 (m, 3H,  $J = 7.48$  Hz, Ar-H), 6.13 (s, 1H, H4 pyrazole), 2.46 (s, 3H, CH3), 2.33 (s, 3H, CH3).  $^{13}\text{C}$  NMR (DMSO, 100MHz): 13.54, 14.18, 105.78, 108.78, 123.07, 125.24, 126.71, 127.51, 128.18, 129.79, 133.19, 135.52, 137.50, 140.92, 150.66, 152.82, 154.90, 156.59. MS (m/z): 381 ( $\text{C}_{22}\text{H}_{19}\text{N}_7$ , 60.53%,  $\text{M}^+$ ), 286 ( $\text{C}_{17}\text{H}_{12}\text{N}_5$ , 100 %). Anal. Calc. for: ( $\text{C}_{22}\text{H}_{19}\text{N}_7$ ) (M.W. = 381): C, 69.27; H, 5.02; N, 25.70%; Found: C, 69.42; H, 5.29; N, 26.09 %.

### General procedure for preparation of compounds IXa-b

To a solution of compound VI (0.001mol) in butanol (20mL), appropriate isocyanates or isothiocyanates (0.001mol) namely, ethyl isothiocyanates, propyl isothiocyanates, was added dropwise at 0°C. The mixture was stirred for specific time at room temperature. Then, the solvent was evaporated under reduced pressure and crude product was purified by crystallization from methanol to yield the corresponding compounds VIII-IX, respectively.

#### ***N*-ethyl-2-(1-phenyl-3-(phenylamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)hydrazine-1-carbothioamide (IXa)**

Yellow solid, Yield: 70%; m.p. 225 °C. IR (KBr)  $\text{cm}^{-1}$ : 3317 (NH), 3015 (CH aromatic), 2965 (CH aliphatic).  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 11.79 (s, 1H, NH), 9.89 (s, 1H, NH), 9.30 (s, 1H, pyrimidine-H2), 8.48 (d, 2H,  $J = 7.88$  Hz, Phenyl-H2,H6), 8.44 (s, 1H, NH), 8.11 (d, 2H,  $J = 7.88$  Hz, NHPhenyl-H2H6), 7.39-7.33 (m, 6H, 5H Ar-H, NH), 6.95 (t, 1H,  $J = 7.32$  Hz, NHPhenyl-H4), 3.58 (q, 2H,  $J = 7.89$  Hz, NHCH2), 1.19 (t, 3H,  $J = 7.79$  Hz, CH2CH3).  $^{13}\text{C}$  NMR (DMSO- $d_6$ , 400 MHz)  $\delta$  (ppm): 15.19, 37.73, (aliphatic carbons), 116.62, 117.25, 117.43, 117.85, 120.18, 120.90, 121.29, 121.04, 122.58, 125.73, 129.10, 129.46, 141.73, (Aromatic carbons), 145.63 (C=S). MS (m/z): 404 ( $\text{C}_{20}\text{H}_{20}\text{N}_8\text{S}$ , 100%,  $\text{M}^+$ ), 360 ( $\text{C}_{18}\text{H}_{14}\text{N}_7\text{S}$ , 32.47%). Anal. Calc. for: ( $\text{C}_{20}\text{H}_{20}\text{N}_8\text{S}$ ) (M.W = 404): C, 59.39; H, 4.98; N, 27.70%; Found: C, 59.19; H, 4.68; N, 27.61%.

#### ***N*'-(1-Phenyl-3-(phenylamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)-*N*-propylhydrazine-1-carbothioamide (IXb)**

Yellow solid, Yield: 65%; m.p. 235 °C. IR (KBr)  $\text{cm}^{-1}$ : 3417 (NH), 3005 (CH aromatic), 2985 (CH aliphatic).  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 13.81 (s, 1H, NH), 11.77 (s, 1H, NH), 10.50 (s, 1H, pyrimidine-H2), 9.65 (d, 2H,  $J = 7.88$  Hz, Phenyl-H2,H6), 8.51 (s, 1H, NH), 8.08 (d, 2H,  $J = 7.88$  Hz, NHPhenyl-H2H6), 7.84 (t, 2H,  $J = 7.72$  Hz, Phenyl-H3,H5), 7.39-7.33 (m, 4H, 3aromatic, NH), 7.02 (t, 1H,  $J = 7.32$  Hz, NHPhenyl-H4), 3.85 (t, 2H,  $J = 7.88$  Hz, NHCH2), 1.62 (m, 2H,  $J = 7.88$  Hz, CH2CH3), 1.22 (t, 3H,  $J = 7.88$  Hz, CH2CH3).  $^{13}\text{C}$  NMR (DMSO- $d_6$ , 400 MHz)  $\delta$  (ppm): 11.35, 22.45, 45.83, (aliphatic carbon), 93.07, 116.62, 117.86, 120.39, 121.29, 122.62, 125.40, 129.10, 139.46, 141.36, 142.12, 144.60, 145.15, (Aromatic carbons), 149.83 (C=S). MS (m/z): 418 ( $\text{C}_{21}\text{H}_{22}\text{N}_8\text{S}$ , 100%,  $\text{M}^+$ ), 360 ( $\text{C}_{18}\text{H}_{14}\text{N}_7\text{S}$ , 38.17%). Anal. Calc. for: ( $\text{C}_{21}\text{H}_{22}\text{N}_8\text{S}$ ) (M.W. = 418): C, 60.27; H, 5.30; N, 26.77%; Found: C, 60.35; H, 5.24; N, 26.62%.

#### ***N*'-(1-phenyl-3-(phenylamino)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)benzohydrazide (X)**

A solution of compound 14 (0.3, 0.001mol) and the benzoyl chloride (0.001mol) in DMF (20mL) in presence of catalytic amount of TEA (0.5mL) was stirred at room temperature for specific time then poured onto ice-cold water. The obtained precipitate was filtered, washed with water, dried and crystallized from glacial acetic acid to afford compound X.

White solid, Yield: 65%; m.p. 256 °C. IR (KBr)  $\text{cm}^{-1}$ : 3417 (NH), 3005 (CH aromatic), 1681 (C=O);  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 10.94 (s, 1H, NH), 8.84 (s, 1H, NH), 8.26 (s, 1H, pyrimidine-H2), 8.19 (d, 2H,  $J = 6.64$  Hz, Phenyl-H2,H6), 8.14 (s, 1H, NH), 7.84 (d, 2H,  $J = 7.96$  Hz, COPhenyl-H2,H6), 7.69 (d, 2H,  $J = 7.56$  Hz, NHPhenyl-H2,H6), 7.52-7.16 (m, 8H,  $J = 7.48$  Hz, aromatic), 6.83 (t, 1H,  $J = 7.16$  Hz, NHPH-H4);  $^{13}\text{C}$  NMR (DMSO, 100MHz): 95.28, 117.43, 121.05, 121.18, 126.56, 127.39, 129.30, 129.69, 130.64, 134.29, 138.82, 141.43, 144.94, 147.91, 150.95, 153.19, 157.62, 164.41 (Aromatic carbons). MS (m/z): 421 ( $\text{C}_{24}\text{H}_{19}\text{N}_7\text{O}$ , 58.76 %,  $\text{M}^+$ ), 286 ( $\text{C}_{17}\text{H}_{12}\text{N}_5$ , 100 %). Anal. Calc. for: ( $\text{C}_{24}\text{H}_{19}\text{N}_7\text{O}$ ) (M.W. = 421): C, 68.40; H, 4.54; N, 23.26%; Found: C, 68.65; H, 4.84; N, 23.62%.

### **2-((1-Phenyl-3-(phenylamino)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)isoindoline-1,3-dione (XI)**

To a solution of VI (0.3, 0.001mol) in glacial acetic acid (20mL), phthalic anhydride (0.15g, 0.001mol) was added. The mixture was heated under reflux for 16h. After completion of the reaction mixture, it was concentrated and allowed to cool. The separated solid was filtered and crystallized from methanol to produce the target compound XI.

Yellow solid, Yield: 62%; m.p. 280 °C. IR (KBr)  $\text{cm}^{-1}$ : 3436 (NH), 3014 (CH aromatic), 1715(CH aliphatic).  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 8.85 (s, 1H, NH), 8.74 (s, 1H, NH), 8.36 (s, 1H, pyrimidine-H2), 8.22 (d, 2H,  $J = 6.64$  Hz, Phenyl-H2,H6), 7.74-7.40 (m, 4H,  $J = 7.96$  Hz, isoindol-H4,H5,H6,H7), 7.35 (d, 2H,  $J = 7.56$  Hz, NHPhenyl-H2,H6), 7.28 (t, 2H,  $J = 6.64$  Hz, Phenyl-H3,H5), 7.22-7.12 (m, 3H,  $J = 7.48$  Hz, aromatic), 6.87 (s, 1H, NHphenyl-H4);  $^{13}\text{C}$  NMR (DMSO, 100MHz): 101.13, 121.61, 122.84, 124.19, 125.19, 126.04, 127.72, 128.81, 130.56, 131.66, 133.57, 135.09, 136.50, 151.12, 152.10, 154.56, 156.15. MS (m/z): 447 ( $\text{C}_{25}\text{H}_{17}\text{N}_7\text{O}_2$ , 48.76 %,  $\text{M}^+$ ), 286 ( $\text{C}_{17}\text{H}_{12}\text{N}_5$ , 100 %). Anal. Calc. for: ( $\text{C}_{25}\text{H}_{17}\text{N}_7\text{O}_2$ ) (M.W. = 447): C, 67.11; H, 3.83; N, 21.91%; Found: C, 67.45; H, 4.14; N, 22.12%.

### **3-(2-(1-Phenyl-3-(phenylamino)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)hydrazono)indolin-2-one(XII)**

To a solution of VI (0.3, 0.001mol) in glacial acetic acid (20mL), isatin (0.14g, 0.001mol) was added. Then, the mixture was heated under reflux for 24h. The reaction mixture was concentrated and allowed to cool. The separated solid was filtered and recrystallized from glacial acetic acid to give the target compound XII.

Orange solid, Yield: 66%; m.p. 278 °C. IR (KBr)  $\text{cm}^{-1}$ : 3412 (NH), 3024 (CH aromatic). 1681 (C=O);  $^1\text{H}$ NMR (DMSO- $d_6$ )  $\delta$  ppm: 10.44 (s, 1H, NH), 9.84 (s, 1H, NH), 8.74 (s, 1H, NH), 8.46 (s, 1H, pyrimidine-H2), 8.24 (d, 2H,  $J = 6.64$  Hz, Phenyl-H2,H6), 7.92-7.36 (m, 11H,  $J = 7.48$  Hz, Ar-H), 6.93 (t, 1H,  $J = 7.16$  Hz, NHPH-H4);  $^{13}\text{C}$  NMR (DMSO, 100 MHz): 101.31, 113.51, 115.54, 120.04, 121.94, 123.07, 124.09, 126.44, 127.81, 128.66, 129.62, 131.06, 132.57, 134.09, 137.50, 140.63, 144.92, 151.89, 153.96, 155.56, 158.61. MS (m/z): 446 ( $\text{C}_{25}\text{H}_{18}\text{N}_8\text{O}$ , 78.76 %,  $\text{M}^+$ ), 286 ( $\text{C}_{17}\text{H}_{12}\text{N}_5$ , 100 %). Anal. Calc. for: ( $\text{C}_{25}\text{H}_{18}\text{N}_8\text{O}$ ) (M.W. = 446): C, 67.25; H, 4.06; N, 25.10%; Found: C, 67.95; H, 4.34; N, 25.52%.

## 5-Conclusion:

A series of novel pyrazolo[3,4-d]pyrimidine derivatives have been designed, and synthesized in useful yields. All the new synthesized compounds were biologically screened in vitro for their cytotoxic activities against a panel of four cancer cell lines namely, HepG-2, MCF-7, HCT-116, and Hela. The results of cytotoxic evaluation indicated that compound VI was found to be the most prominent broad-spectrum cytotoxic activity and significantly activity with IC<sub>50</sub> values of 6.18, 6.48, 4.03, and 5.82 μM against tested cell lines. In addition, compound IXb displayed promising cytotoxic effect against all tested cell lines with IC<sub>50</sub> value less than 30 μM compared with doxorubicin as a control drug. Pharmacophoric features indicated that pyrazolo[3,4-d]pyrimidine scaffold having a four atoms linker was more potent than those possessing other linkers which lead to significant decrease in cytotoxic activity.

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### تشبيد وتقييم بيولوجي لبعض مشتقات البيرازولوبيريبيدين الجديده المتوقع لها نشاط مضاد للسرطان

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#### الملخص :

تم تصميم وتشبيد سلسله من مشتقات البيرازولوبيريبيدين الجديده فى انتاجية مفيده صناعيا من خلال بعض المركبات الوسيطة وتم اثبات الصيغ البنائيه للمركبات باستعمال جهاز الاشعه دون الحمراء وجهاز الرنين النووى المغناطيسى ومطياف الكتله هذا بالاضافه الى التحليل الدقيق لعناصر المركبات التى تبين نسبة الكربون والهيدروجين والنيتروجين فى المركبات وقد تم تقييم جميع المركبات بيولوجيا فى المختبر لانشطتها السامه للخلايا السرطانيه فى بعض انواع السرطان كسرطان الكبد والثدى والقولون والحنجره.

وتم اجراء الاختبارات البيولوجيه على المركبات الجديده فوجد ان لها تاثير مضاد للسرطان وذلك بالمقارنه بمضاد مرجعي للسرطان مثل (دوكسوروبيسين).

وقد اظهرت نتائج تقييم السمييه لهذه المركبات على النحو التالى بالنسبه للنشاط المضاد للسرطان فقد كانت اقوى النتائج هى للمركب السادس حيث اظهر التأثير الابرز ضد جميع الخلايا السرطانيه المختبره بنتائج تتراوح بين ٤.٠٣ الى ٦.٤٨ ميكرومول بالمقارنه بقيم نتائج الدوكسوروبيسين كعقار محكمالى تتراوح بين ٤.٥ الى ٥.٥٧ ميكرومول بينما اظر المركبات التاسع الف وباء قيما متوسطه تتراوح بين ١٤.٧٦ الى ٣٠.٦ ميكرومول بالمقارنه بالدوكسوروبيسين اما باقى المركبات فكانت نتائجها اقل قوه مقارنة بالمركبات الاخرى.

**الكلمات المفتاحيه :** مثبطات مستقبل عامل نمو البشره، مشتقات البيرازولو بيريبيدين ، مضاد للسرطان