



# Design, synthesis and biological evaluation of certain CDK2 inhibitors based on pyrazole and pyrazolo[1,5-a] pyrimidine scaffold with apoptotic activity

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## ABSTRACT

Different series of novel pyrazole and pyrazolo[1,5-a] pyrimidine derivatives (**2a-g**), (**3a-c**), (**7a-d**) and (**10a-e**) were designed, synthesized and evaluated for their ability to inhibit CDK2/cyclin A2 enzyme *in vitro*. In addition, the cytotoxicity of the newly synthesized compounds was screened against four different human cancer cell lines. The CDK2/cyclin A2 enzyme inhibitory activity revealed that compounds (**2d**) and (**2g**) are among the most active with inhibitory activity values of 60% and 40%, respectively, while compounds (**7d**) and (**10b**) exhibited the highest activity among the newly synthesized derivatives against four tumor cell lines (HepG2, MCF-7, A549 and Caco2) with IC50 values 24.24, 14.12, 30.03 and 29.27  $\mu\text{M}$  and 17.12, 10.05, 29.95 and 25.24  $\mu\text{M}$ , respectively. Flow cytometry cell cycle assay was carried for compounds (**7d**) and (**10b**) to investigate their apoptotic activity. The obtained results revealed that they induced cell-cycle arrest in the G0-G1 phase and reinforced apoptotic DNA fragmentation. Molecular modeling studies have been carried out to gain further understanding the binding mode of the target compounds together with field alignment to define the similar field properties.

## 1. Introduction

Protein kinases constitute a large group of evolutionary and structurally related enzymes that regulate the function of other proteins through the catalysis of the transfer of the  $\gamma$  phosphate group from a nucleoside triphosphate (ATP), to the side chain of an amino acid residue in the substrate protein; serine, threonine, histidine or tyrosine residues [1]. Protein phosphorylation plays a critical role in the regulation of cellular function [2].

Based upon their catalytic specificity, kinases can be categorized into: (I) Tyrosine kinases (TK) [3], (II) Serine/Threonine kinases [4] and (III) Histidine Kinases [5]. The cyclin dependent kinases (CDK) (a serine/threonine kinase family) [6] are responsible for the initiation and the succession of each cell cycle phase; CDK2 activity is required for progression through G1 to the S phase [7]. CDK2 hyper activation in human cancers is often associated with amplification and/or over-expression of its partner cyclins A and E in a diverse of human cancers particularly in breast, ovarian, endometrial, lung and thyroid carcinomas, melanoma and osteosarcoma [8]. Pyrazoles and pyrazolo pyrimidines, especially pyrazolo[1,5-a]pyrimidines, represent common nucleus in many pharmaceutical active compounds that have a wide range of significant pharmacological activities especially the inhibition

of CDK2/Cyclin A [9–11] as shown in Fig. 1.

In this research, the design of the target compounds with the pyrazole core (**2a-g**) and (**3a-c**) was derived from the structure optimization of the reference compound AT7519 (**IV**) based on its reported structure activity relationship SAR [14,15] as following: Figs. 2 and 3.

- The core pyrazole nucleus of the lead compound AT7519 (**IV**) that occupy the adenine region of ATP binding pocket is maintained in our target compounds.
- AT7519 (**IV**) is anchored to the hinge region of CDK2 through a donor–acceptor donor H-bonding with the backbone GLU81 and LEU83. This interaction is retained in compounds (**2a-g**) via the pyrazole ring (N2 & CO) and the NH of the amino methylene group at position 4 of pyrazole ring. While compounds (**3a-c**) exhibit donor- acceptor H-bond interaction only.
- The amino piperidine amide group of AT7519 (**IV**) that improved the hydrophobic filling of the region bounded by the backbone of the, was replaced at position 4 of the pyrazole ring by; a) substituted phenyl amino methylene groups in compounds (**2a-g**), b) substituted phenyl furanyl methylene groups in compounds (**3a-b**) and c) substituted benzylidene group in compound (**3c**).
- The 2, 6-dichlorobenzamide of AT7519 (**IV**) with the two chlorine

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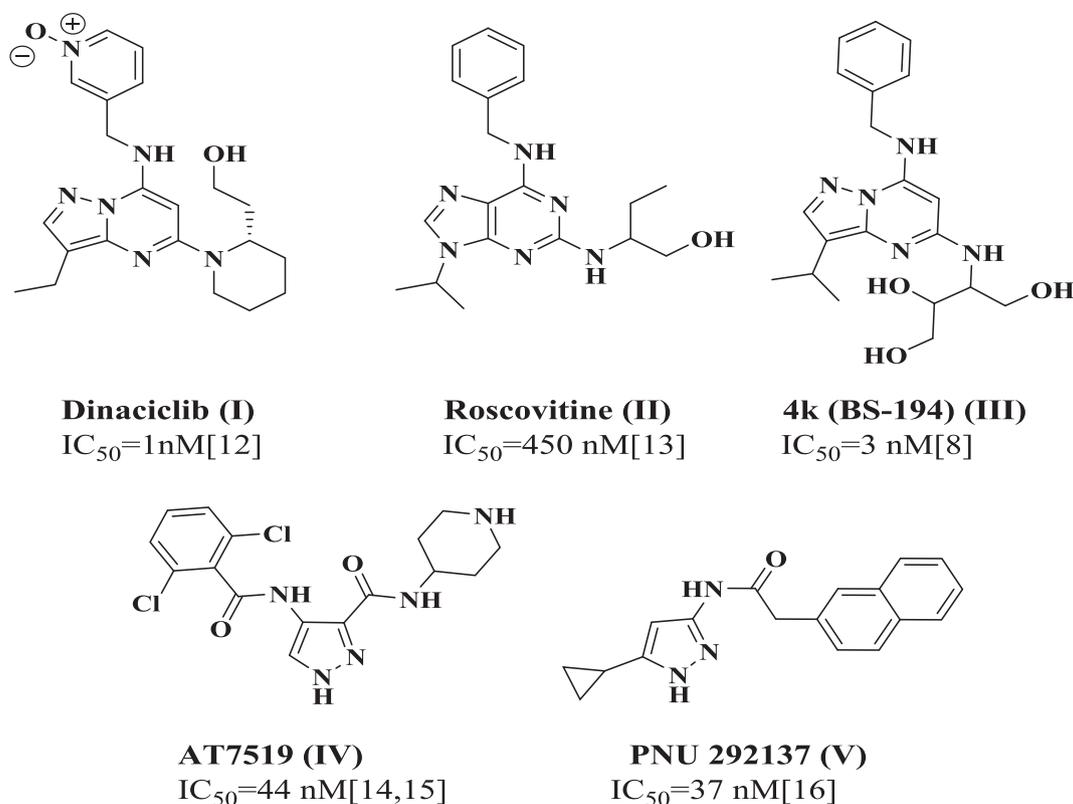


Fig. 1. The Chemical structure for clinically approved CDK2 inhibitors (I-V).

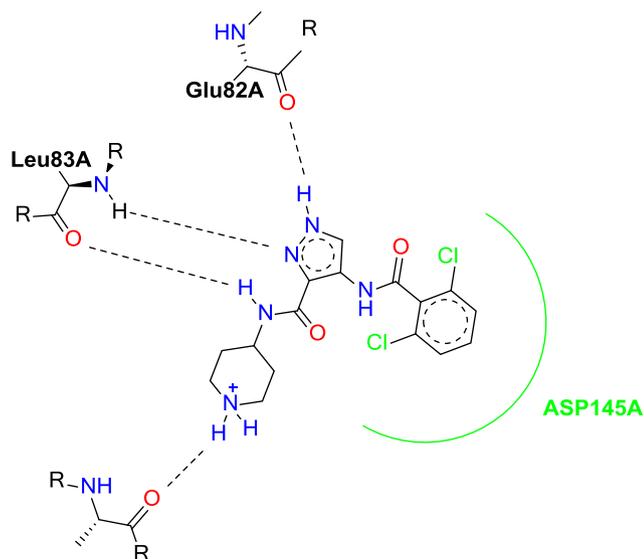


Fig. 2. Schematic illustration of the binding mode of the reference compound AT7519 (IV) to the active site of CDK2 (2H-bond with LEU 83).

atoms efficiently filling small hydrophobic pockets, was swapped with phenyl amino group at position 5 of the pyrazole ring to occupy this hydrophobic pocket.

The design the pyrazolo[1,5-*a*]pyrimidine scaffold ((7a-d) and (10a-e)) was based on some structural modifications of the lead compounds Roscovitine (II) and 4 k (BS-194) (III) [8,13] as shown in Figs. 4 and 5.

- Purine of Roscovitine (II) that occupies approximately the same region as the purine ring of ATP was bioisosterically replaced by

pyrazolo[1,5-*a*]pyrimidine nucleus in the reference compounds 4 k (BS-194) (III), so it becomes an elegant nucleus of choice.

- The hydroxylated ethylamino and propylamino substitutions at C<sup>2</sup> of Roscovitine (II) and the three side chain hydroxyl groups of 4 k (BS-194) (III) are essential for hydrophilic interaction in the ribose binding site. This substitutions were changed to carboxylic acid ethyl ester group at position 6 of the pyrazolo[1,5-*a*]pyrimidine nucleus.
- The N<sup>6</sup> benzyl amino substituent of Roscovitine (II) provides  $\pi$ - $\pi$  interaction between with the amino acid residues ILE10, PHE82 and HIS84. While the N<sup>7</sup> benzyl amino substituent of 4 k (BS-194) (III) occupies a hydrophobic pocket outside the ATP binding site. In the newly designed compounds, this substitution was replaced by phenyl amino substituent but at position 2 of the pyrazolo[1,5-*a*]pyrimidine nucleus.
- The isopropyl substituent at N<sup>9</sup> of Roscovitine (II) or at position 3 of 4 k (BS-194) (III) was replaced at position 3 of the pyrazolo[1,5-*a*]pyrimidine nucleus with hydrophilic carbamide or cyanide moiety in compounds (7a-d) and (10a-e), respectively, to provide extra H-bonding interaction.
- The introduction of hydrophobic group as substituted phenyl group at position 5 of the pyrazolo[1,5-*a*]pyrimidine nucleus aiming to provide additional hydrophobic interaction.

## 2. Results and discussion

### 2.1. Chemistry

Final compounds incorporating substituted methylene and amino methylene moieties were obtained utilizing 5-phenylamino-2,4-dihydro-pyrazol-3-one (1) [16], which was synthesized according to the route outlined in (Scheme 1a) via nucleophilic addition reaction of the highly reactive C<sup>2</sup> of ethyl acetoacetate to phenyl isothiocyanate, to give the sodium salt of 2-(Mercapto-phenylamino-methylene)-3-oxo-

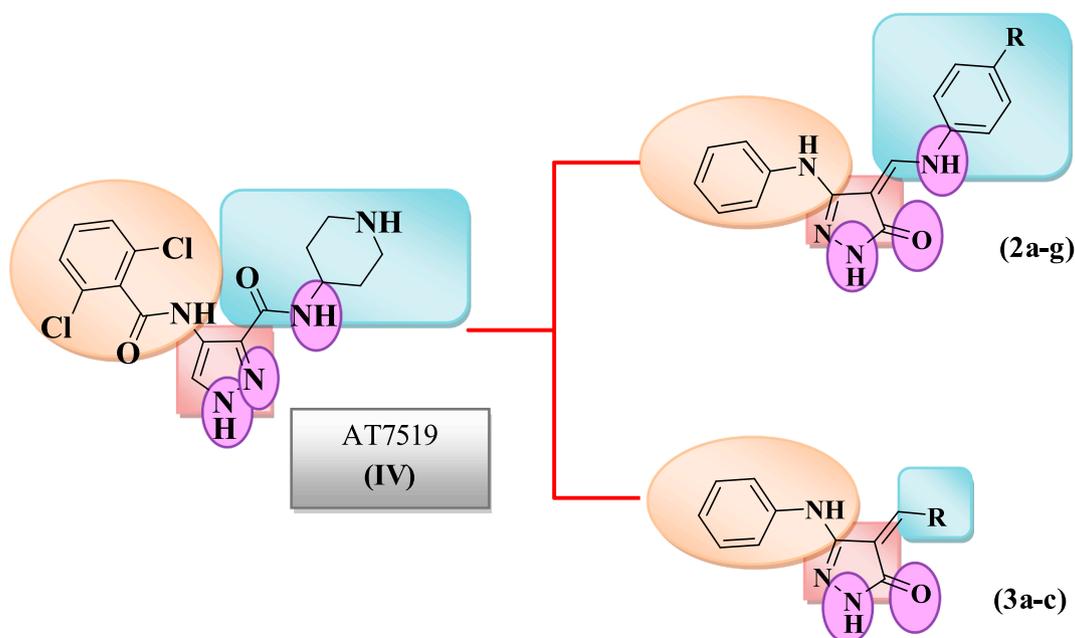


Fig. 3. Similarities of pharmacophoric features between the reference compound AT7519 (IV) and the target compounds (2a-g) and (3a-c).

butyric acid ethyl ester. Cyclization took place through the addition of hydrazine hydrate to the previous alcoholic reaction mixture.

The synthesis of the target compounds (2a-g) was achievable via the fusion of the 5-phenylamino-2,4-dihydro-pyrazol-3-one (1) with the appropriate aryl amine and triethyl orthoformate in DMSO for 2.5–4.5hrs, afforded the corresponding 4-[(4-substituted-phenylamino)-methylene]-5-phenylamino-2,4-dihydro-pyrazol-3-one (2a-g) with a good yield (Scheme 1b). According to Knoevenagel condensation reaction [17,18], the nucleophilic addition reaction of C<sup>4</sup> of 5-phenylamino-2,4-dihydro-pyrazol-3-one (1) to the corresponding aldehyde, followed by the dehydration reaction affording the corresponding (4-substituted) methylene-5-phenylamino-2,4-dihydro-pyrazol-3-one (3a-c) (Scheme 1b).

The synthesis of the pyrazolo[1,5-a]pyrimidine target compounds (7a-d) was initiated via the preparation of the key cyanoacrylamide intermediate (5), by nucleophilic addition reaction of C<sup>2</sup> of cyanoacetamide to phenyl isothiocyanate followed by the methylation reaction using dimethyl sulphate [19–21]. The later was then cyclized directly upon the fusion with hydrazine hydrate [22] to afford 5-amino-3-phenylamino-1H-pyrazole-4-carboxylic acid amide (6), which in turn was

refluxed with different aromatic aldehydes and a  $\beta$ -ketoester (ethyl acetoacetate) in DMF to afford the final target compounds (7a-d) through biginelli reaction [23,24] (Scheme 2).

Whilst similar synthetic procedures were adopted for the synthesis of the pyrazolo[1,5-a]pyrimidine target compounds (10a-e). Malononitrile was our key intermediate instead of the cyanoacrylamide and gave 2-(Methylsulfonyl-phenylamino-methylene)-malononitrile (8). The later was then cyclized directly upon the fusion with equimolar amount of hydrazine hydrate to afford 5-Amino-3-phenylamino-1H-pyrazole-4-carbonitrile (9), which in turn was refluxed with different aromatic aldehydes and a  $\beta$ -ketoester (ethyl acetoacetate) in DMF to afford the final target compounds (10a-e).

The newly synthesized derivatives (2a-g) revealed spectral analysis data matched with their structures. The IR spectra of compounds (2a-g) showed the appearance of extra absorption band of NH around  $3100\text{ cm}^{-1}$ , while compound (2e) illustrated the existence of two extra absorption bands; one for NH at  $3873\text{ cm}^{-1}$  and the other for CO at  $1743\text{ cm}^{-1}$ . Whereas compound (2f) demonstrated one extra absorption band of CO at  $1743\text{ cm}^{-1}$ . Compound (2g) proved the appearance of two absorption bands; one for NH at  $3032\text{ cm}^{-1}$  and for the other for

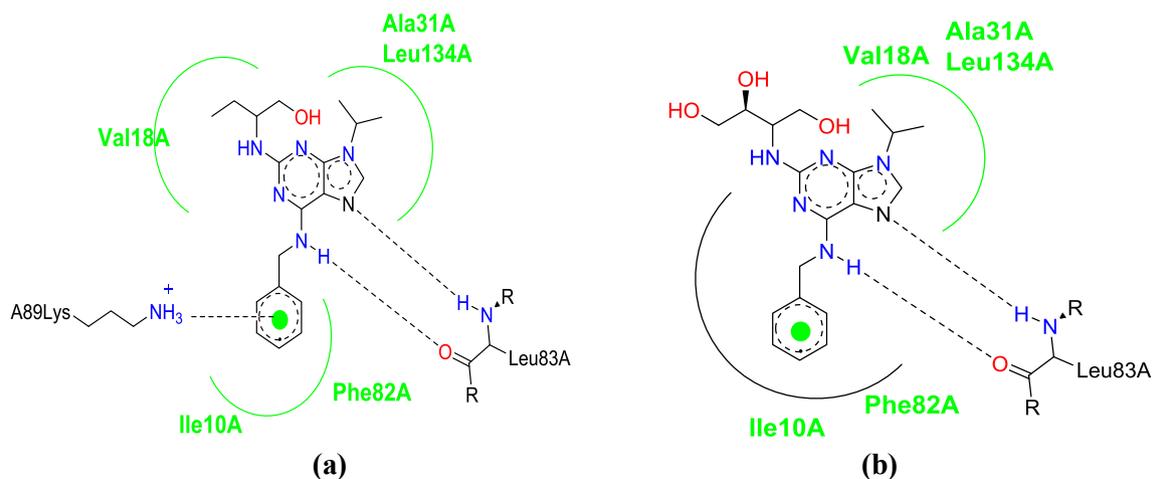


Fig. 4. Schematic illustration of the binding modes of the reference compounds (a) Roscovitine (II) and (b) 4k (BS-194) (III) to the active site of CDK2 (2H-bond with LEU 83).

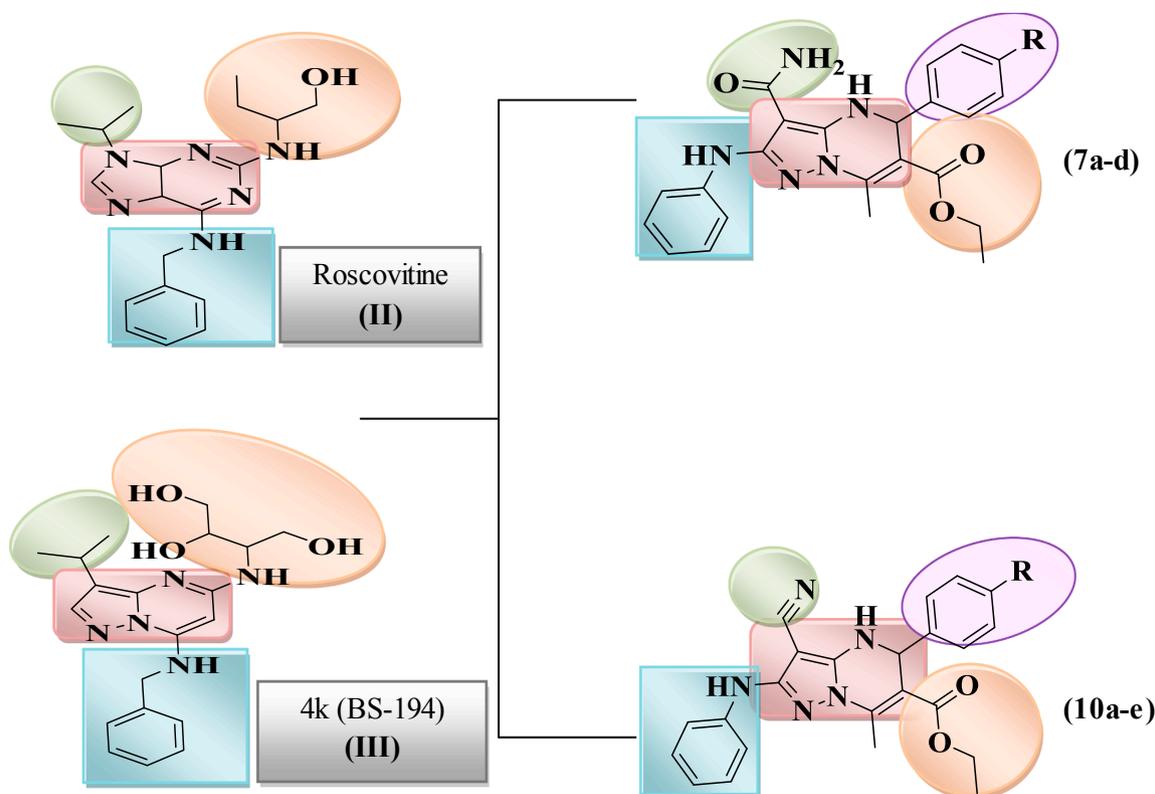


Fig. 5. Similarities of the pharmacophoric features among the reference compounds Roscovitine (II), 4k (BS-194) (III) and the target compounds (7a-d) and (10a-e).

SO<sub>2</sub>-N at 1377 cm<sup>-1</sup>. (see Scheme 3)

The <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) spectra of the synthesized compounds (2a-g) exemplified the presence of extra aromatic protons at the range of δ 7.4 to 7.6 ppm and an extra olefinic singlet proton around δ 8.7 ppm. While compounds (2d), (2e) & (2g) illustrated the appearance of an extra singlet exchangeable proton by D<sub>2</sub>O of Ar-OH at δ 9.47 ppm, Amide-NH at δ 10.49 ppm and Ar-NH at δ 9.79 ppm, respectively. The spectra of the produced compounds (2c), (2e) & (2f) exhibited the existence of one singlet peak around δ 2.5 ppm for the CH<sub>3</sub> group and compound (2g) showed the appearance of one singlet overlapped peak at δ 2.6 ppm of the two CH<sub>3</sub> groups. The <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) spectrum of the produced compound (2c) confirmed the appearance of an absorption peak at δ 30.61 ppm for the added CH<sub>3</sub> group.

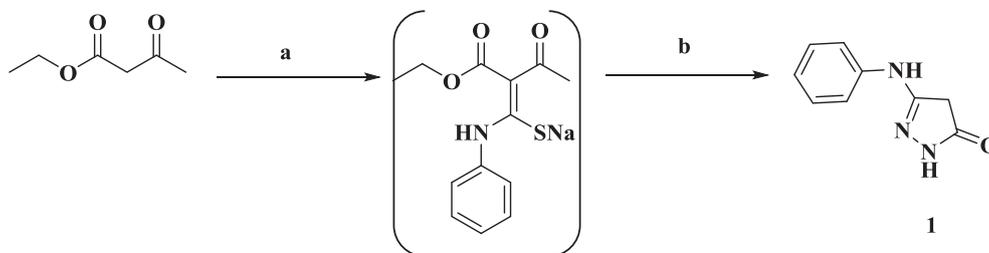
For compounds (7a-d), their IR spectra displayed the disappearance of one absorption band of NH that was present in the intermediate (6) and the existence of extra CO band around 1700 cm<sup>-1</sup>. While, compound (7c) illustrated additional absorption band of OH at 3425 cm<sup>-1</sup>.

Even as the <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) spectra of the synthesized compounds (7a-d) demonstrated the presence of extra aliphatic protons of Pyrimidinyl-CH<sub>3</sub>, CH<sub>3</sub>CH<sub>2</sub> and CH<sub>3</sub>CH<sub>2</sub> groups at the range of δ 2.5 to 4.14 ppm and the appearance of singlet proton of

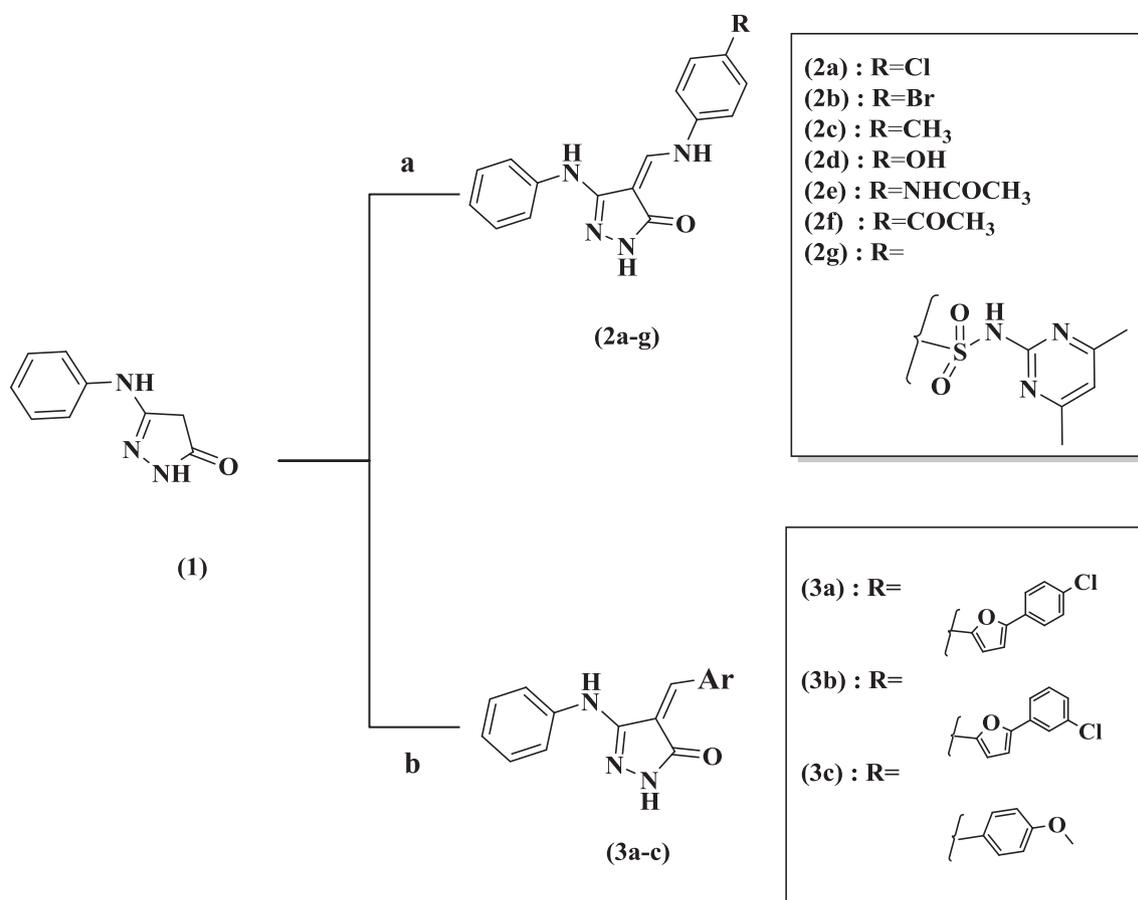
Pyrimidinyl-H around δ 5.5 ppm. Compound (7b) showed the presence of extra aliphatic singlet proton of OCH<sub>3</sub> group at δ 3.85 ppm. Where, compound (7c) proved the presence of extra singlet exchangeable proton by D<sub>2</sub>O of OH at δ 9.58 ppm. The <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) spectrum of the produced compound (7b) showed the appearance of absorption peak at δ 55.59 ppm of the added OCH<sub>3</sub> group.

The structure of compounds (10a-e) was elucidated on the basis of spectral data. The IR spectra showed the disappearance of one absorption band of NH was present in the intermediate (9) and the appearance of CO band around 1680 cm<sup>-1</sup>. Compound (10c) illustrated the appearance of OH band at 3429 cm<sup>-1</sup>.

While the synthesized compounds (10a-e) showed the presence of extra aliphatic protons of Pyrimidinyl-CH<sub>3</sub>, CH<sub>3</sub>CH<sub>2</sub> and CH<sub>3</sub>CH<sub>2</sub> groups at the range of δ 2.4 to 4.1 ppm and the appearance of singlet proton of Pyrimidinyl-H around δ 5.4 ppm in their <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) spectra. Compound (10b) showed the presence of extra aliphatic singlet proton of OCH<sub>3</sub> group at δ 3.68 ppm. While, compound (10e) showed the presence of one singlet aliphatic proton of N(CH<sub>3</sub>)<sub>2</sub> at δ 2.82 ppm. The <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) spectrum of the produced compound (10b) showed the appearance of absorption peak at δ 55.6 ppm the added OCH<sub>3</sub> group. The mass analysis of all compounds was consistent.



Scheme 1a. Reagents and conditions: (a) Na, CH<sub>3</sub>OH, phenyl isothiocyanate, reflux 1/2 hr, cool (b) Hydrazine hydrate, reflux 1 hr, 72–75%.



**Scheme 1b.** Reagents and conditions: (a) Aryl amine, triethyl orthoformate, DMSO, Oil Bath, 2.5–4.5 hrs, 81–85% (b) Aromatic aldehyde, sodium acetate, AcOH, reflux 6 hrs, 78–81%.

### 3. Biological evaluation

#### 3.1. *In vitro* CDK2/cyclin A2 activity

The CDK2/cyclin A2 protein kinase assays were performed at BPS Bioscience, San Diego, CA, USA ([www.bpsbioscience.com](http://www.bpsbioscience.com)) using Kinase-Glo Plus luminescence kinase assay kit (Promega). It measures kinase activity by quantitating the amount of ATP remaining in solution following a kinase reaction. The luminescent signal from the assay is correlated with the amount of ATP present and is inversely correlated with the amount of kinase activity. The percent inhibition of the tested compounds against CDK2/cyclin A2 protein kinase was compared to reference kinase inhibitor dinaciclib at a single concentration of 10  $\mu$ M. The computer software Graphpad Prism was used for analyzing the luminescence data. The difference between luminescence intensities in the absence of CDK2/cyclin A2 protein kinase ( $Lu_t$ ) and in the presence of kinase ( $Lu_c$ ) was defined as 100% activity ( $Lu_t - Lu_c$ ). Using luminescence signal ( $Lu$ ) in the presence of the compound, % activity was calculated as:

- % activity =  $\{(Lu_t - Lu)/(Lu_t - Lu_c)\} \times 100\%$ , where  $Lu$  = the luminescence intensity in the presence of the compound. % Inhibition was calculated as: % inhibition =  $100 (\%) - \% \text{ activities}$ .

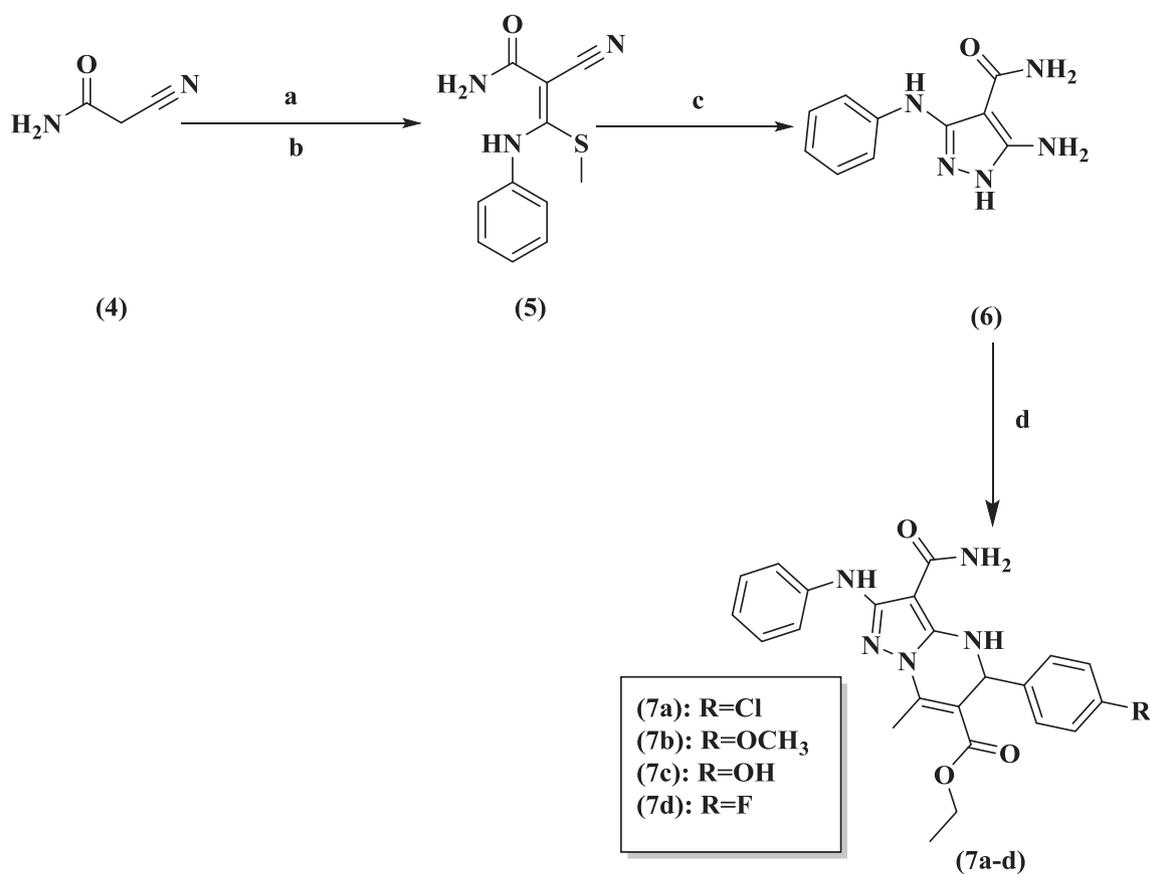
An initial screening; all synthesized final compounds were evaluated for their inhibitory activity against CDK2/cyclin A2 protein kinase at a single dose concentration of 10  $\mu$ M. At this concentration, two compounds (**2d**) and (**2g**) of the pyrazole series had the highest competitive inhibitory effect among the newly synthesized derivatives with % inhibitory values 60% and 44%, respectively. Nevertheless, the

pyrazole derivatives (**3a**) & (**3c**) and the pyrazolo[1,5-*a*]pyrimidine derivatives (**7c**), (**10a**), (**10b**), (**10c**) & (**10d**) showed low to moderate competitive inhibitory effect with % activity changes values ranging from 10% to 28%. The mean percent CDK2/cyclin A2 protein kinase inhibition of the investigated compounds at 10  $\mu$ M concentration are shown in (Table 1). (Dinaciclib [12] as a reference compound showed 100% CDK2/cyclin A2 protein kinase inhibition).

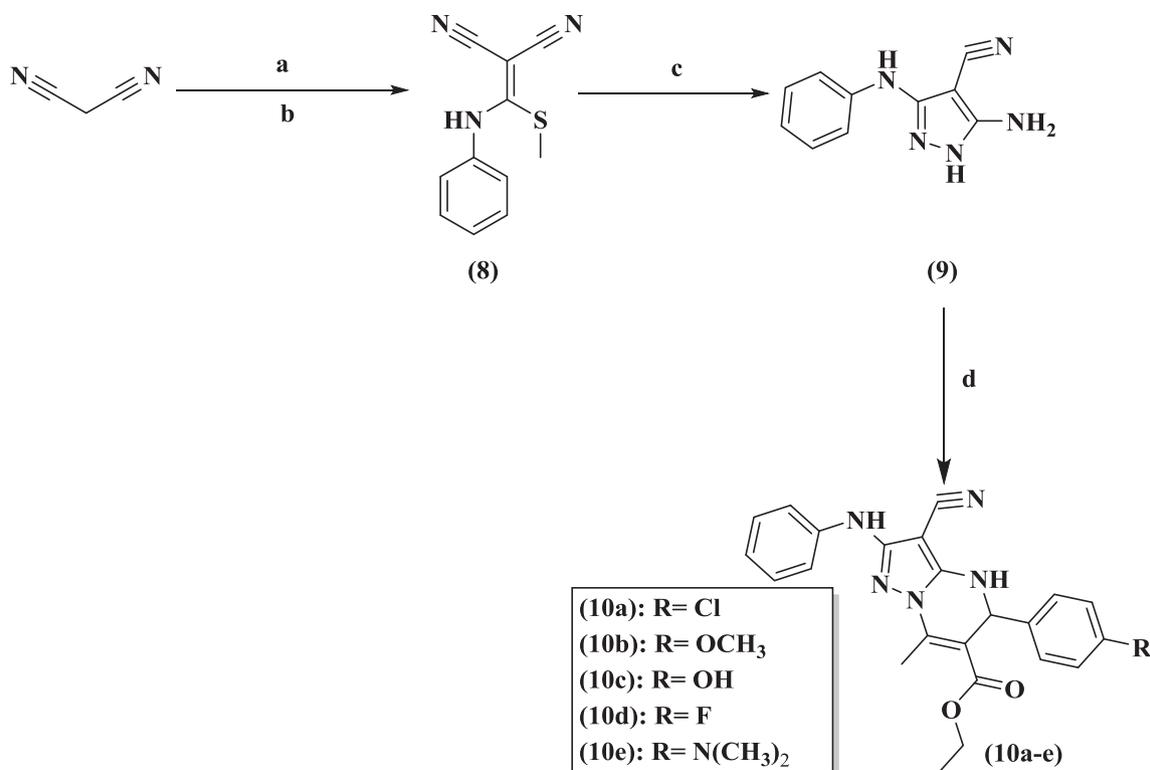
#### 3.2. *In vitro* antiproliferative activity.

Cytotoxicity assays were conducted to determine the level of sensitivity as well as selectivity of cancer and normal cells to the targeted compounds. All the newly synthesized compounds (**2a-g**), (**3a-c**), (**7a-d**) and (**10a-e**) were screened for their anticancer activity against four different human tumor cell lines including human liver cancer cell line (HepG2), human breast cancer cell line (MCF-7), human lung cancer cell line (A549) and human colon cancer cell line (Caco2) at pharmacology department of Al- Azhar University, Egypt, using MTT colorimetric assay according to Mosmann [25]. This test is based on the selective ability of living cells, not dead cells, to reduce the yellow soluble salt of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple-blue insoluble formazan precipitate, this dye is accomplished by the mitochondrial dehydrogenases of viable cells. The viable cell number is proportional to the production of formazan salts.

The experiments were performed in six replicates for each compound and the results were normalized to the control value and expressed as percentage of control. The compound concentrations which give 50% growth inhibition are referred to as the IC<sub>50</sub>. The anticancer activity was determined using Doxorubicin [26] as a reference (Table 2).

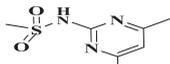
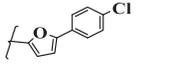
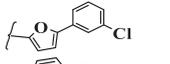
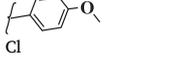


**Scheme 2.** Reagents and conditions: (a) KOH, DMF, phenyl isothiocyanate, stirring, RT, 24 hrs (b) Dimethyl sulphate, stirring, RT, 8 hrs, 80% (c) Hydrazine hydrate, W. B., 3–4 hrs, 64% (d) Hydrochloric acid, aromatic aldehyde, ethyl acetoacetate, ethanol, reflux, 4–8 hrs 80–83%.



**Scheme 3.** Reagents and conditions: (a) KOH, DMF, phenyl isothiocyanate, stirring, RT, 24 hrs (b) Dimethyl sulphate, stirring, RT, 8 hrs, 82% (c) Hydrazine hydrate, W. B., 3–4 hrs, 80% (d) Hydrochloric acid, aromatic aldehyde, ethyl acetoacetate, ethanol, reflux, 4–8 hrs 80–83%.

**Table 1**  
Inhibition activity results (% Inhibition) of the synthesized compounds against CDK2/cyclin A2.

Cpd ID	R	%Inhibition
(2a)	Cl	6
(2b)	Br	4
(2c)	CH <sub>3</sub>	8
(2d)	OH	60
(2e)	NHCOCH <sub>3</sub>	8
(2f)	COCH <sub>3</sub>	9
(2g)		44
(3a)		11
(3b)		8
(3c)		11
(7a)	Cl	5
(7b)	OCH <sub>3</sub>	3
(7c)	OH	28
(7d)	F	6
(10a)	Cl	12
(10b)	OCH <sub>3</sub>	10
(10c)	OH	11
(10d)	F	13
(10e)	N(CH <sub>3</sub> ) <sub>2</sub>	9

**Table 2**  
In vitro cytotoxic activity of the newly synthesized compounds and doxorubicin against MCF-7, HepG2, A549 and Caco-2 tumor cell lines.

Cpd	MCF-7 IC <sub>50</sub> μM	HepG2 IC <sub>50</sub> μM	A549 IC <sub>50</sub> μM	Caco-2 IC <sub>50</sub> μM
<b>Doxorubicin</b>	0.44	0.977	5.842	8.508
(2a)	346.01	> 500	> 500	> 500
(2b)	97.64	76.34	112.11	79.78
(2c)	30.96	34.92	61.34	58.22
(2d)	23.32	37.24	41.42	50.3
(2e)	42.19	79.02	90.35	88.26
(2f)	92.72	123	120	146
(2g)	334.47	> 500	> 500	265.35
(3a)	133.1	184.7	192.1	160.2
(3b)	100.1	120.92	160.5	208.1
(3c)	30.06	37.91	42.90	52.12
(7a)	132.5	160.4	185.8	352
(7b)	39.09	72.52	111.22	88.14
(7c)	30.09	66.12	58.54	99.91
(7d)	14.12	24.24	30.03	29.27
(10a)	35.53	62.35	71.23	90.35
(10b)	10.05	17.12	29.95	25.24
(10c)	30.06	69.44	82.15	41.07
(10d)	30.47	69.35	81.44	59.96
(10e)	86.23	125.24	150.4	157.9

The analysis of the IC<sub>50</sub> results in (Table 2) disclosed that:

Compounds (7d) and (10b) revealed the highest activity against the four tumor cell lines [(HepG2), (MCF-7), (A549) and (Caco2)] among the newly synthesized derivatives with IC<sub>50</sub> values for (7d) are 24.24, 14.12, 30.03 and 29.27 μM and for (10b) are 17.12, 10.05, 29.95 and 25.24 μM, respectively. Whereas, compounds (2c), (2d), (7c), (10c) and (10d) divulged comparable anticancer activity against (MCF-7) cancer cell line with IC<sub>50</sub> values 30.96, 23.32, 30.09, 30.06 and 47 μM, respectively.

Derivatives (2c), (2d) and (3c) showed moderate antiproliferative activity against three tumor cell lines [(HepG2), (A549) and (Caco2)] with IC<sub>50</sub> values ranging from 34.92 to 61.34 μM. Rather though this moderate activity has been also displayed against (MCF-7) cancer cell line by compounds (2e), (7b) and (10a) with IC<sub>50</sub> values ranging from

35.53 to 42.19.34 μM. Where, compounds (10c) and (10d) exhibited well to moderate antiproliferative activity against (Caco2) cancer cell line with IC<sub>50</sub> values 41.07 and 59.96 μM, respectively.

### 3.3. Flow cytometry cell cycle analysis

Cell cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of the cell cycle. Before analysis, the cells are permeabilised and treated with a fluorescent dye that stains DNA quantitatively, as Propidium Iodide (PI). The fluorescence intensity of the stained cells will therefore correlate with the amount of DNA they contain. As the DNA content of cells duplicates during the S phase of the cell cycle, the relative amount of cells in the G<sub>0</sub> phase and G<sub>1</sub> phase (before S phase), in the S phase, and in the G<sub>2</sub> phase and M phase (after S phase) can be determined, since the fluorescence of G<sub>2</sub>/M cells is twice as high as that of G<sub>0</sub>/G<sub>1</sub> cells.

Cell staining with PI is considered as univariate measurement of the cellular DNA content alone. This approach reveals the distribution of cells in three major phases of the cycle (G<sub>1</sub> vs S vs G<sub>2</sub>/M) and makes it possible to detect apoptotic cells with fractional DNA content. It utilizes Propidium Iodide (PI) as the DNA fluorochrome and requires blue light as the excitation source (e.g., 488 nm argon ion laser). Because PI also stains double-stranded RNA, the latter is removed by the addition of RNase A to the staining solution [27].

Since compounds (7d) and (10b) showed the highest anticancer activity against the four tumor cell lines and represent different series that were synthesized in this work, so they were selected to test their effect on cell cycle of HCT-116 cells. The results are presented in the following (Table 3).

As shown in Fig. 6, the assay outcomes proved that upon exposure HCT-116 cells to compounds (7d) and (10b), a significant increase in the percentage of the sub-G<sub>0</sub>/G<sub>1</sub> cells by 67.37% and 65.43%, respectively occurred when they were compared to the cells of control sample with 47.91%. Moreover, (10b) causes cell cycle arrest at S phase by 24.68% in comparison to control sample with 20.85%. A sub-G<sub>0</sub>/G<sub>1</sub> peak represents the relative amount of apoptotic cells in the sample. These results demonstrated that compounds (7d) and (10b) inhibit the proliferation of HCT-116 cells and cause apoptotic DNA fragmentation.

## 4. Molecular modeling studies

### 4.1. Docking study

Molecular modeling simulation study was performed through docking of the target compounds in the binding site of CDK2 enzyme using C-Docker protocol in Discovery Studio 2.5 Software. In this investigation, docking study on the target compounds and analysis of their binding modes was performed to interpret the biological results and to gain further insight into binding orientations and interactions. The selected docking pose among the 10 retrieved possible ones was chosen based on the similarity of its binding mode to that of the co-crystallized ligand.

It is worth noting that both the X-ray crystallographic enzyme (CDK2) substrate complex with AT7519 (IV) (PDB code 2VU3) and with Roscovitine (II) (PDB code 2A4L) revealed the formation of the essential two H-bonds with LEU83. Validation of C-Docker protocol used

**Table 3**

The percentage of HCT-116 cells in G<sub>0</sub>-G<sub>1</sub> vs. S vs. G<sub>2</sub>/M after being treated with compounds (7d) and (10b) in comparison to control (untreated cells).

Sample	%G <sub>0</sub> -G <sub>1</sub>	%S	%G <sub>2</sub> -M
Control	47.91	20.85	31.24
(7d)	67.37	18.6	14.03
(10b)	65.43	24.68	9.89

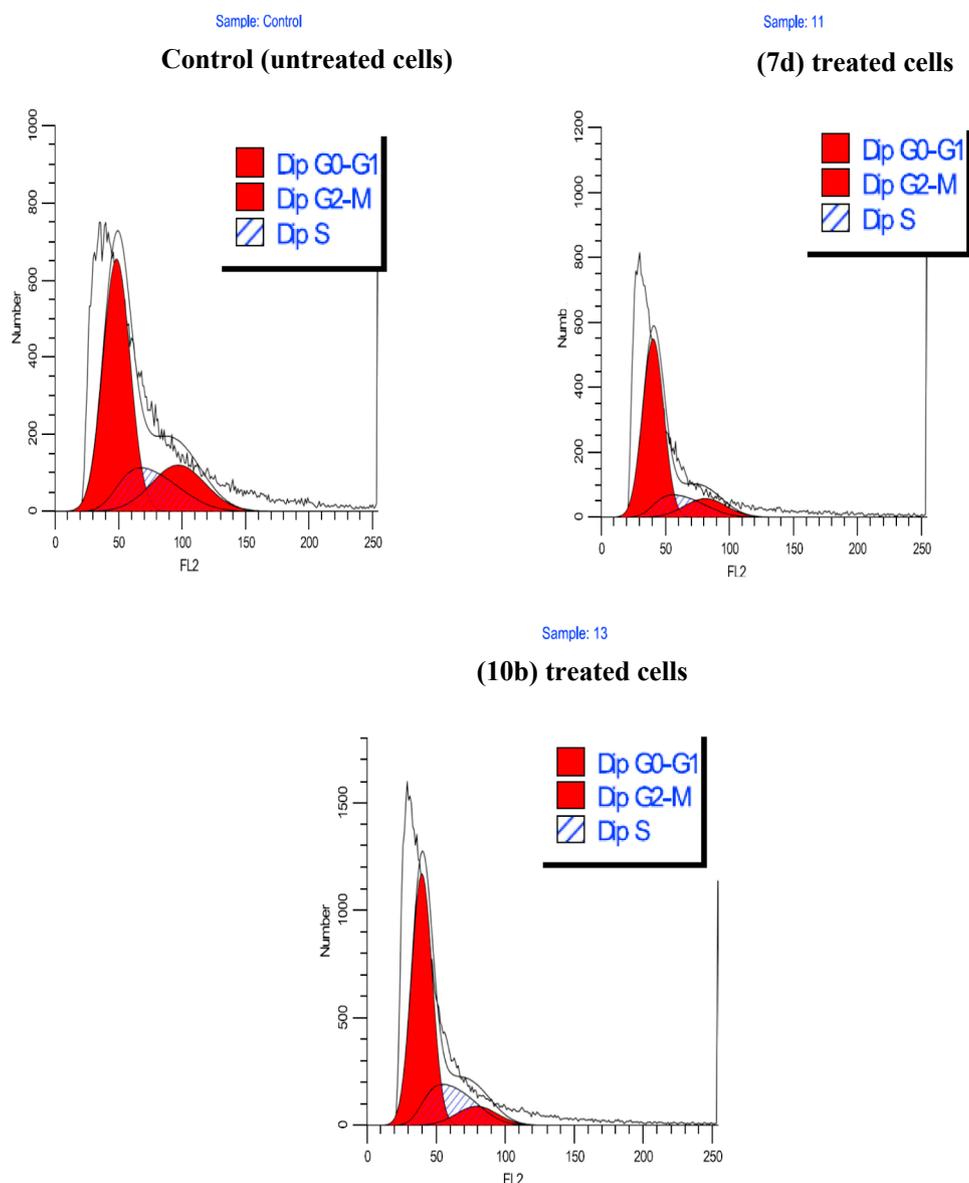


Fig. 6. Cell cycle assay and effect of compounds (7d) and (10b) on HCT-116 cells in comparison to control (untreated cells) evaluated by flow cytometry.

in this study was performed by re-docking the lead compounds in the CDK2 kinase active site. This was followed by the alignment of the X-ray bioactive conformer of the lead compound with the best fitted pose achieved from the docking run. The alignment showed good coincidence between them with RMSD = 0.5 Å, indicating the ability of the used docking protocol to retrieve valid docking poses.

The docking study of the synthesized compounds into CDK2 active site revealed comparable binding modes of the docked molecules to the lead compound. The binding interactions of the docked compounds together with their binding energies are presented below in Fig. 7. By studying the CDocker interaction and the binding mode of the biologically active compounds (2d), (2g), (7d) and (10b), we figured out that:

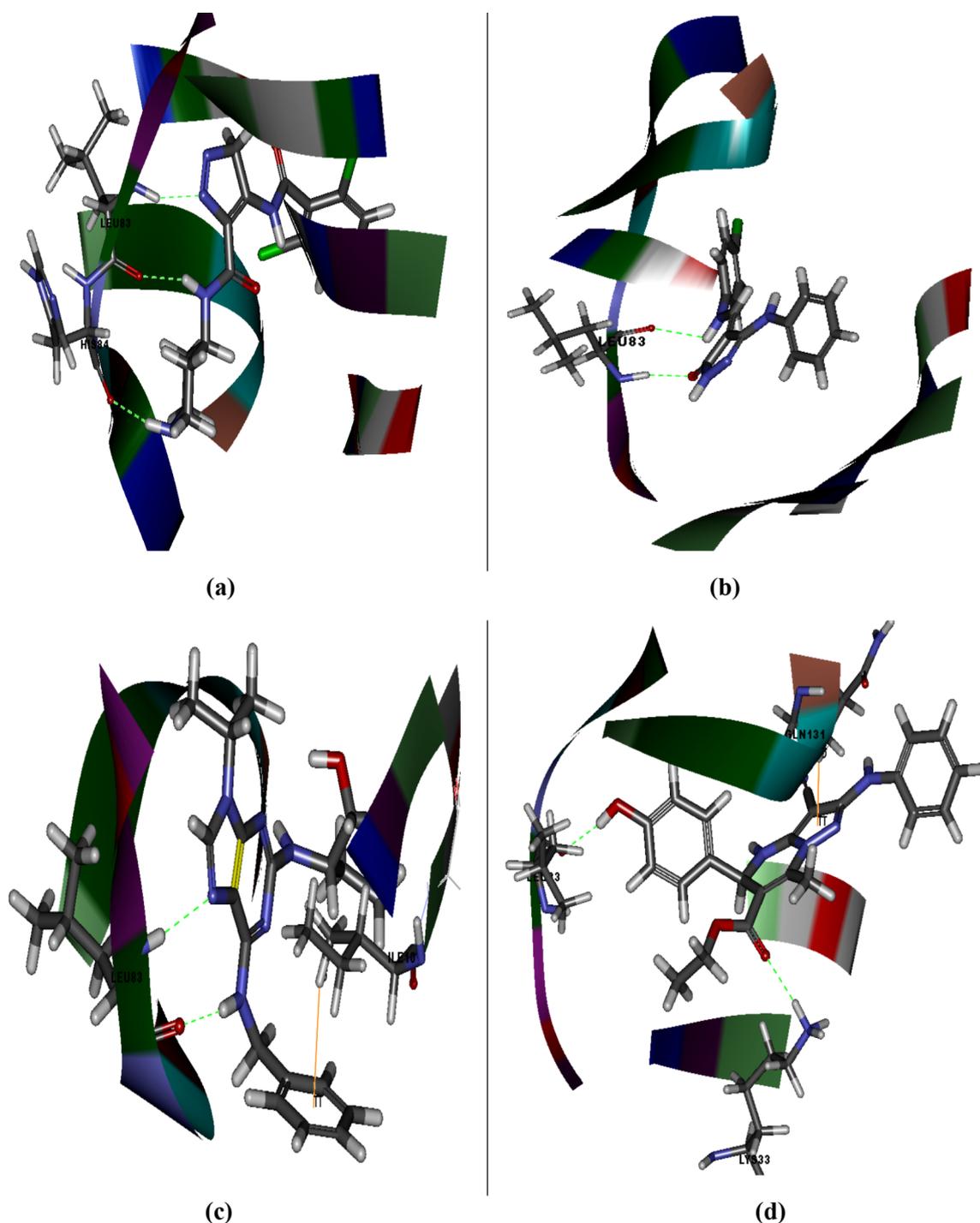
Compound (2d) conserved the formation of the two H bonds with LEU83 of the reference compound AT7519 (IV) and exhibited high antitumor activity against (MCF-7) cancer cell line with high % of inhibition against CDK2/A2 which proves that it's antitumor activity due to the inhibition of CDK2 enzyme and can be used as a lead for further studies.

Whereas compound (2g) formed only one H bond with LEU83 and

showed different binding mode other than the reference compound AT7519 (IV). Compounds (7d) and (10b) didn't form this essential H-bonding with LEU83 other than the reference compounds Roscovitine (II) and (4k) (BS-194) (III), but they exhibited new binding mode to the active site relative to these reference compounds.

#### 4.2. Field alignment study methodology.

2D structure of the reference compounds [AT7519 (IV) and (4k) (BS-194) (III)] and the target compounds were constructed using ChemDraw Ultra®. The 2D structures were subjected a conformational search MMFF94x forcefield applying the default settings. The energy minimized structures were saved in files with the appropriate extensions. FieldAlign was used to align the structures of the target compounds to the reference compounds. The most accurate mode was used in the settings. FieldAlign, conformation hunter algorithm was used to generate representative sets of conformations corresponding to local minima of energy calculated within the extended electron distribution force field. Superimpositions of the conformations to the template were refined by the simplex optimization algorithm incorporated in



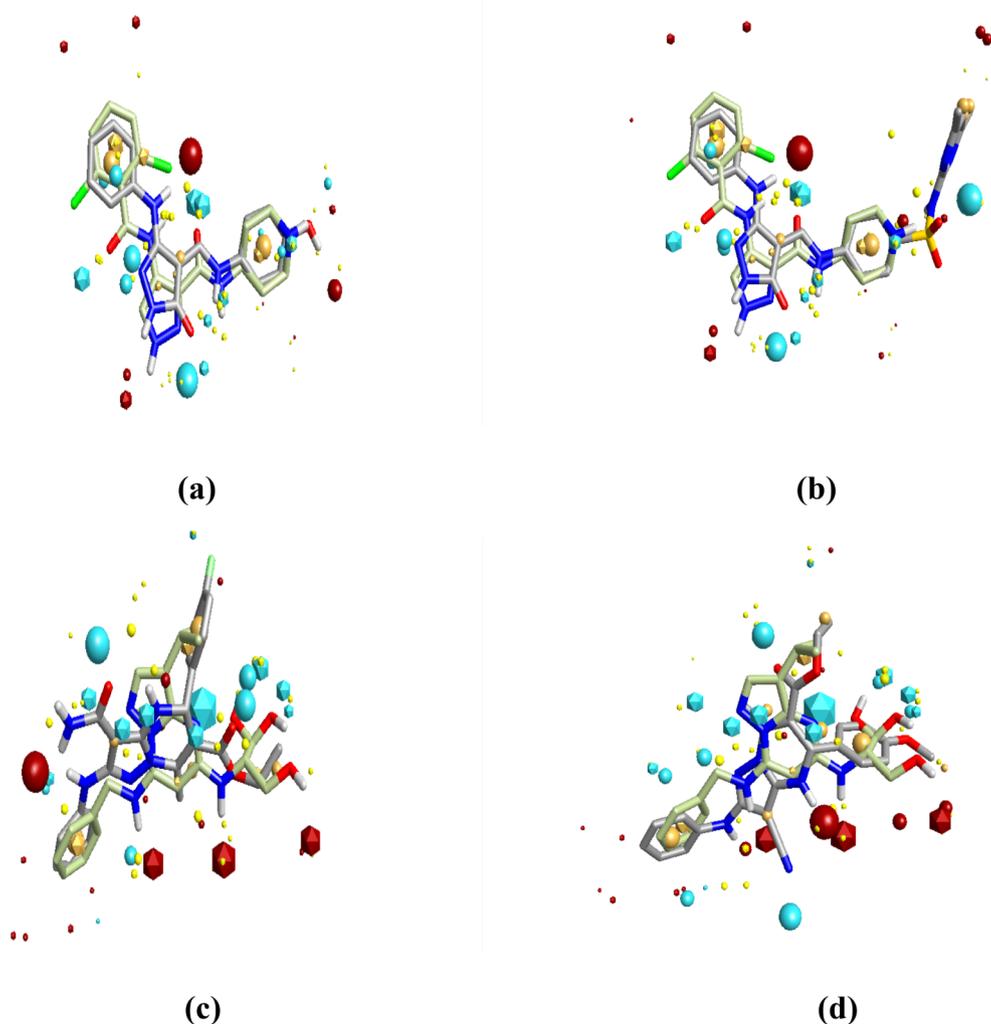
**Fig. 7.** (a) Retrieved docking pose of the pyrazole-based inhibitor (IV) (PDB code 2VU3) showing the same key interactions as reported. (b) Docking pose of the target compounds (2a) to the ATP binding pocket of CDK2 in its inactive conformation. (c) Retrieved docking pose of the pyrazolo[1,5-a]pyrimidine based inhibitor (II) (PDB code 2A4L) showing the same key interactions as reported. (d) Docking pose of the target compounds (10b) to the ATP binding pocket of CDK2 in its inactive conformation.

#### FieldAlign Fig. 8.

Compound (2d) showed good alignment with approximately the same field points of the reference compound AT7519 (19), whereas compound (2g) illustrated different alignment other than the reference compound AT7519 (IV) due to the steric sulfonamide moiety. Compounds (7d) and (10b) didn't exhibit good alignment with the reference compound (4k) (BS-194) (III), but they showed different field points in the active site relative to the reference compound, therefore they displayed lower CDK2/cyclin A2 affinity.

#### 5. Conclusion

A new series of pyrazole and pyrazolo[1,5-a]pyrimidine derivatives (2a-g), (3a-c), (7a-d) and (10a-e) were designed to act as anticancer via CDK2 inhibition. The promising candidates were synthesized and evaluated as inhibitors of CDK2/cyclin A2. Compounds (2d) and (2g) with the highest inhibitory activity against CDK2/cyclin A2 with % activity values 60% and 44%, respectively which showed high docking scores and low binding energy values and their binding mode was



**Fig. 8.** (a& b) the best aligned poses of compounds (2d) & (2g) (grey) overlaid with the reference ligand AT7519 (IV) (light green), respectively. (c & d) The best aligned poses of compounds (7d) & (10e) (grey) overlaid with the reference ligand (4k) (BS-194) (III) (light green), respectively.

compatible with the reported binding mode of reference compound AT7519 (IV).

Two compounds (7d) and (10b) exhibited high anticancer activity against four tumor cell lines [(HepG2), (MCF-7), (A549) and (Caco2)] among the newly synthesized series with IC<sub>50</sub> values for (7d) are 24.24, 14.12, 30.03 and 29.27  $\mu$ M and for (10b) are 17.12, 10.05, 29.95 and 25.24  $\mu$ M, respectively, but they showed different binding mode and field points rather than the reference compounds in the docking and field alignment study.

Compounds (7d) and (10b) were selected to test their effect on cell cycle of HCT-116 cells by Propidium Iodide (PI) flow cytometric assay; they cause a significant increase in the percentage of the sub-G<sub>0</sub>/G<sub>1</sub> cells by 67.37% and 65.43%, respectively when they were compared to the cells of control sample with 47.91%. This peak reveals that compounds (7d) and (10b) inhibit the proliferation of HCT-116 cells and cause apoptotic DNA fragmentation.

## 6. Experimental

Starting materials, reagents and solvents were purchased from Sigma-Aldrich (USA) or Alfa- Aesar Organics and used without further purification. Reactions were monitored by analytical thin layer chromatography (TLC), performed on silica GF254 plates packed on Aluminium sheets, purchased from (E. Merck, Germany) with visualization under U.V. light (254 nm). Melting points ( $^{\circ}$ C) were determined

by open capillary tube method using (XT4 MP) apparatus and they are uncorrected. Mass spectrum was carried out on Direct Inlet part to mass analyzer in Thermo Scientific GCMS model ISQ at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Nasr City, Cairo. The <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Varian spectrometer 300 MHz, at the Micro-analytical Data Center of Cairo University, or 500 MHz, at National Research Center, at 25  $^{\circ}$ C with TMS and solvent signals allotted as internal standards. All coupling constant (*J*) values are given in Hertz. Chemical shifts ( $\delta$ C) are reported relative to DMSO-*d*<sub>6</sub> as internal standards. The abbreviations used are as follows: s: singlet; d: doublet; t: triplet; q: quartet and m: multiplet. Analytical data (IR) was performed at NODCAR, the Micro-analytical Data Center of Cairo University and National Research Center. Elemental analyses were performed on a CHNS-O-Rapid instrument in the Micro-analytical Data Center at Cairo University.

Thin layer chromatography was performed on pre-coated (0.25 mm) silica gel GF254 plates (E. Merck, Germany), compounds were detected with 254 nm UV lamp. Silica gel (60–230 mesh) was employed for routine column chromatography separations.

### 6.1. Chemistry

The synthesis of the reported intermediates is presented in [Supplementary Materials](#).

### 6.1.1. General procedure for preparation of 5-Phenylamino-2,4-dihydro-pyrazol-3-one (1) [16]

To a solution of sodium methylate (8.8 gm of sodium in 120 ml of methanol), (50 gm, 0.38 mol) of ethyl acetoacetate was added and adjoined with (52 gm, 0.38 mol) of phenyl isothiocyanate in a thin stream at room temperature. The mixture was refluxed for  $\frac{1}{2}$  hr, cooled to about 50 °C, so it was ready for hydrazine hydrate addition in the next step. Hydrazine hydrate 85% (11.9 ml, 0.38 mol) added in a thin stream with stirring to the previous reaction mixture. After refluxing for 1 hr, the mixture was cooled, filtered and the product was washed and crystallized from ethanol and gave white crystals of the title compound (72%).

**6.1.1.1. 5-Phenylamino-2,4-dihydro-pyrazol-3-one (1).** hite powder; yield (72%); m.p.: 268–270 °C;  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 3.41 (s, 2H, Pyrazole-CH<sub>2</sub>), 6.74 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.15 (t, 1H, Ar-H), 7.43 (d, 2H, overlapped, Ar-H), 9.16 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 10.35 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 175.19)  $m/z$ , 175.07 (M<sup>+</sup>, [100%]).

### 6.1.2. General procedure for preparation of 4-[(4-substituted-phenylamino)-methylene]-5-phenylamino-2,4-dihydro-pyrazol-3-one (2a-g) [28,29]

A mixture of 5-phenylamino-2,4-dihydro-pyrazol-3-one (1) (0.8 gm, 0.005 mol) and appropriate aryl amine (0.005 mol) was fused with triethyl orthoformate (0.66 gm, 0.005 mol) in oil bath at 140–160 °C for 2.5–4.5 hrs. The resulting precipitate was washed well via diethyl ether, filtered and crystallized from ethanol to give to the tilted compounds (2a-g).

**6.1.2.1. 4-[(4-Chloro-phenylamino)methylene]-5-phenylamino-2,4-dihydro-pyrazol-3-one (2a).** Brown powder; (85%); m.p.: > 300 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3421 (NH), 3232 (NH), 3140 (NH), 1670 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 6.84 (t, 1H, Ar-H), 7.26 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.43 (d, 2H,  $J$  = 8.7HZ, Ar-H), 7.51 (d, 2H, overlapped, Ar-H), 7.61 (d, 2H,  $J$  = 8.5HZ, Ar-H), 8.55 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 8.71(s, 1H, Olefinic-CH), 10.54 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 11.1 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 312.75):  $m/z$ , 312.11 (M<sup>+</sup>, [100%]), 314.09 (M<sup>+2</sup>, [39.77%]); **Anal.** Calcd. for C<sub>16</sub>H<sub>13</sub>ClN<sub>4</sub>O; C, 61.44; H, 4.19; N, 17.91; Found: C, 61.73; H, 4.21; N, 18.11.

**6.1.2.2. 4-[(4-Bromo-phenylamino)methylene]-5-phenylamino-2,4-dihydro-pyrazol-3-one (2b).** Orange powder; yield (82%); m.p.: 285–287 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3440 (NH), 3238 (NH), 3143 (NH), 1671 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 6.82 (t, 1H, Ar-H), 7.23 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.34 (d, 2H,  $J$  = 8.6HZ, Ar-H), 7.56 (d, 2H,  $J$  = 8.7HZ, Ar-H), 7.61 (d, 2H,  $J$  = 8.4HZ, Ar-H), 8.51 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 8.68 (s, 1H, Olefinic-CH), 10.50 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 11.1 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 357.20):  $m/z$ , 356.07 (M<sup>+</sup>, [100%]), 358.08(M<sup>+2</sup>, [95.67%]); **Anal.** Calcd., for C<sub>16</sub>H<sub>13</sub>BrN<sub>4</sub>O; C, 53.80; H, 3.67; N, 15.68; Found: C, 54.11; H, 3.85; N, 15.91.

**6.1.2.3. 5-Phenylamino-4-(4-tolylamino-methylene)-2,4-dihydro-pyrazol-3-one (2c).** Brownish orange powder; yield (84%); m.p.: 293–295 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3427 (NH), 3242(NH), 3145 (NH), 1671 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.46 (s, 3H, Ar-CH<sub>3</sub>), 6.81 (t, 1H, Ar-H), 7.21 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.27 (d, 2H,  $J$  = 8.7HZ, Ar-H), 7.4 (d, 2H,  $J$  = 8.6HZ, Ar-H), 7.56 (d, 2H,  $J$  = 8.5HZ, Ar-H), 8.49 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 8.71 (s, 1H, Olefinic-CH), 10.46 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 11.11 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O);  $^{13}\text{C NMR}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 30.61, 107.35, 115.18, 115.31, 115.54, 115.65, 118.59, 128.66, 129.22, 129.5, 130.31, 130.55, 142.75, 146.61, 149.16, 159.55, 167.20; **MS:** (Mwt.: 292.34):  $m/z$ , 292.17 (M<sup>+</sup>, [100%]); **Anal.** Calcd., for C<sub>17</sub>H<sub>16</sub>N<sub>4</sub>O; C, 69.85; H, 5.52; N, 19.17; Found: C, 70.11; H, 5.75; N, 19.33.

**6.1.2.4. 4-[(4-Hydroxy-phenylamino)-methylene]-5-phenylamino-2,4-dihydro-pyrazol-3-one (2d).** Brown powder; yield (85%); m.p.: 210–215 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3426 (OH), 3392 (NH), 3240 (NH), 3145 (NH), 1665 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 6.8 (t, 1H, Ar-H), 7.18 (d, 2H,  $J$  = 8.7HZ, Ar-H), 7.24 (d, 2H,  $J$  = 8.5HZ, Ar-H), 7.43 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.55 (d, 2H,  $J$  = 8.4HZ, Ar-H), 8.44 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 8.59 (s, 1H, Olefinic-CH), 10.4 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 9.47 (s, 1H, Ar-OH, exchangeable by D<sub>2</sub>O), 11.09 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 294.31):  $m/z$ , 294.17 (M<sup>+</sup>, [14.77%]); **Anal.** Calcd., for C<sub>16</sub>H<sub>14</sub>N<sub>4</sub>O<sub>2</sub>; C, 65.3; H, 4.79; N, 19.04; Found: C, 65.44; H, 4.95; N, 19.21.

**6.1.2.5. N-{4-[(5-Oxo-3-phenylamino-1,5-dihydro-pyrazol-4-ylidene)methyl]-amino}-phenyl acetamide (2e).** Brown powder; yield (81%); m.p.: > 300 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3873 (NH), 3248 (NH), 3140 (NH), 3062 (NH), 1743(CO), 1666 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.5 (s, 3H, Amide-CH<sub>3</sub>), 6.9 (t, 1H, Ar-H), 7.24 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.32 (d, 2H,  $J$  = 8.5HZ, Ar-H), 7.47 (d, 2H,  $J$  = 8.6HZ, Ar-H), 7.63 (d, 2H,  $J$  = 8.7HZ, Ar-H), 8.53 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 8.7 (s, 1H, Olefinic-CH), 9.8 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 10.01 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O), 10.49 (s, 1H, Amide-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 335.36):  $m/z$ , 335.2 (M<sup>+</sup>, [8.35%]); **Anal.** Calcd., for C<sub>18</sub>H<sub>17</sub>N<sub>5</sub>O<sub>2</sub>; C, 64.47; H, 5.11; N, 20.88; Found: C, 64.61; H, 5.39; N, 20.97.

**6.1.2.6. 4-[(4-Acetyl-phenylamino)-methylene]-5-phenylamino-2,4-dihydro-pyrazol-3-one (2f).** Brown powder; yield (85%); m.p.: > 300 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3325 (NH), 3209 (NH), 3093 (NH), 1743 (CO), 1640 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.5 (s, 3H, Acetyl-CH<sub>3</sub>), 6.84 (t, 1H, Ar-H), 7.26 (d, 2H,  $J$  = 8.5HZ, Ar-H), 7.43 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.51 (d, 2H, overlapped, Ar-H), 7.61 (d, 2H,  $J$  = 8.6 HZ, Ar-H), 8.55 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 8.71 (s, 1H, Olefinic-CH), 10.54 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 11.16 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 320.35):  $m/z$ , 320.15 (M<sup>+</sup>, [1.95%]); **Anal.** Calcd., for C<sub>18</sub>H<sub>16</sub>N<sub>4</sub>O<sub>2</sub>; C, 67.49; H, 5.03; N, 17.49; Found: C, 67.61; H, 5.21; N, 17.65.

**6.1.2.7. N-(4,6-Dimethyl-pyrimidin-2-yl) -4-[(5-oxo-3-phenylamino -1,5-dihydro-pyrazol-4-ylidene)methyl]-amino]-benzene sulfonamide (2g).** Black powder; yield (82%); m.p.: > 300 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3367 (NH), 3232 (NH), 3055 (NH), 3032 (NH), 1620 (CO), 1377 (SO<sub>2</sub>-N);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.6 (s, 6H, 2Ar-CH<sub>3</sub>), 6.28 (s, 1H, Pyrimidinyl-CH), 6.53 (t, 1H, Ar-H), 7.15 (d, 2H,  $J$  = 8.4HZ, Ar-H), 7.41 (d, 2H,  $J$  = 8.5HZ, Ar-H), 7.58 (d, 2H,  $J$  = 8.6HZ, Ar-H), 7.64 (d, 2H,  $J$  = 8.7HZ, Ar-H), 7.66 (s, 1H, Olefinic-CH), 8.03 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 9.79 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 10.26 (s, 1H, Ar-NH, exchangeable by D<sub>2</sub>O), 12.85 (s, 1H, Pyrazole-NH, exchangeable by D<sub>2</sub>O); **MS:** (Mwt.: 463.51):  $m/z$ , 463.17 (M<sup>+</sup>, [2.47%]); **Anal.** Calcd., for C<sub>22</sub>H<sub>21</sub>N<sub>7</sub>O<sub>3</sub>S; C, 57.01; H, 4.57; N, 21.15; Found: C, 57.21; H, 4.75; N, 21.31.

### 6.1.3. General procedure for preparation of (4-substituted) methylene-5-phenylamino-2,4-dihydro-pyrazol-3-one (3a-c) [17,18]

A mixture of 5-phenylamino-2, 4-dihydro-pyrazol-3-one 1 (4gm, 0.025 mol) with appropriate aromatic aldehyde (0.025 mol) was refluxed for 6 hrs with freshly fused sodium acetate (7gm) in glacial acetic acid (80 ml). After that, the reaction mixture was cooled and poured on ice where the precipitate occurred. The later was filtered and washed well with water and crystallized from ethanol to give the title compound (3a-c).

**6.1.3.1. 4-[5-(4-Chloro-phenyl)-furan-2-ylmethylene]-5-phenylamino-2,4-dihydro pyrazol-3-one (3a).** Olive green powder; yield (80%); m.p.:128–130 °C; **IR:** ( $\nu$  max, cm<sup>-1</sup>): 3550 (NH), 3478 (NH), 1617(CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 6.91 (d, 2H,  $J$  = 8.7HZ, Ar-H), 7.3 (t, 1H, Ar-H), 7.48 (d, 1H,  $J$  = 8.4HZ, furan-H), 7.59 (d, 2H,

$J = 8.6\text{Hz}$ , Ar-H), 7.68 (d, 1H,  $J = 8.5\text{Hz}$ , furan-H), 7.9 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 8.07 (d, 2H, overlapped, Ar-H), 8.64 (s, 1H, Olefinic-CH), 8.8 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 10.72 (s, 1H, Pyrazole -NH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.:363.8):  $m/z$ , 363.12 ( $\text{M}^+$ , [29.04%]), 365.11 ( $\text{M}^{+2}$ , [14.1%]); **Anal.** Calcd., for  $\text{C}_{20}\text{H}_{14}\text{ClN}_3\text{O}_2$ ; C, 66.03; H, 3.88; N, 11.55; Found: C, 66.21; H, 4.11; N, 11.75.

**6.1.3.2. 4-[5-(3-Chloro-phenyl)-furan-2-ylmethylene]-5-phenylamino-2,4-dihydro pyrazol-3-one (3b)**. Dark green powder; yield (78%); m.p.: 198–200 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3547 (NH), 3413 (NH), 1680 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 6.89 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.15 (t, 1H, Ar-H), 7.28 (d, 1H,  $J = 8.5\text{Hz}$ , furan-H), 7.44 (d, 1H,  $J = 8.5\text{Hz}$ , furan-H), 7.53 (d, 2H,  $J = 8.4\text{Hz}$ , Ar-H), 7.66 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.73 (t, 1H, Ar-H), 7.89 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 8.1 (s, 1H, Ar-H), 8.63 (s, 1H, Olefinic-CH), 9.11 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 10.5 (s, 1H, Pyrazole -NH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 363.80):  $m/z$ , 363.1 ( $\text{M}^+$ , [19.01%]), 365.1 ( $\text{M}^{+2}$ , [7.59%]); **Anal.** Calcd., for  $\text{C}_{20}\text{H}_{14}\text{ClN}_3\text{O}_2$ ; C, 66.03; H, 3.88; N, 11.55; Found: C, 66.21; H, 3.81; N, 11.74.

**6.1.3.3. 4-(4-Methoxy-benzylidene)-5-phenylamino-2,4-dihydro pyrazol-3-one (3c)**. Dark red powder; yield (81%); m.p.: 184–186 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3525 (NH), 3387 (NH), 1678 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 3.86 (s, 3H,  $\text{OCH}_3$ ), 6.91 (d, 2H, overlapped, Ar-H), 7.12 (t, 1H, Ar-H), 7.27 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 7.66 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.9 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 8.63 (s, 1H, Olefinic-CH), 9.8 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 10.68 (s, 1H, Pyrazole -NH, exchangeable by  $\text{D}_2\text{O}$ );  $^{13}\text{C NMR}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 55.59, 114.35, 114.66, 118.6, 126.26, 127.89, 128.3, 128.69, 129.22, 129.55, 140.45, 142.75, 146.7, 149.16, 155.75, 165.20, 169.78; **MS**: (Mwt.: 293.32):  $m/z$ , 293.13 ( $\text{M}^+$ , [31.33%]); **Anal.** Calcd., for  $\text{C}_{17}\text{H}_{15}\text{N}_3\text{O}_2$ ; C, 69.61; H, 5.15; N, 14.33; Found: C, 69.89; H, 5.31; N, 14.55.

#### 6.1.4. General procedure for preparation of cyanoacetamide (4) [30]

Ethyl 2-cyanoacetate (52 ml, 0.442 mol) was poured on ammonium hydroxide (40 ml, 1.14 mol), then stirred until a turbid solution formed. The mixture was gently heated until it turned clear. The mixture has been kept in an ice bath for 1 hr upon which a crystalline white precipitate formed. The precipitate was filtered and crystallized from ethanol to give the title compound as crystalline white powder (70%).

#### 6.1.5. General procedure for preparation of 2-Cyano-3-methylsulfanyl-3-phenylamino-acrylamide (5) [19,20]

To a cooled solution of finely divided KOH (1.65gm, 0.025 mol) in DMF, cyanoacetamide (4) (2.1gm, 0.025 mol) was added then followed by the addition of phenyl isothiocyanate (3.37gm, 0.025 mol). Reaction mixture was stirred at room temperature for 24 hrs then cooled again with addition of water (30 ml). Dimethyl sulphate (3.15gm, 0.025 mol) was added drop wisely and stirred continuously for extra 8 hrs to give a crystalline yellow precipitate. The precipitate was filtered and washed well with ether then crystallized from ethanol to give the title compound (5) (80%).

**6.1.5.1. 2-Cyano-3-methylsulfanyl-3-phenylamino-acrylamide (5)**. Pale yellow powder; yield (80%); m.p.: 147–149 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3382 ( $\text{NH}_2$ ), 3329 (NH), 2225 (CN), 1652 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.51 (s, 3H,  $\text{CH}_3$ ), 7.14 (d, 2H,  $J = 8.4\text{Hz}$ , Ar-H), 7.29 (t, 1H,  $J = 8.7\text{Hz}$ , Ar-H), 7.42 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 12.11 (s, 1H, NH, exchangeable by  $\text{D}_2\text{O}$ ), 12.46 (s, 2H,  $\text{NH}_2$ , exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 233.1):  $m/z$ , 233.05 ( $\text{M}^+$ , [0.78%]).

#### 6.1.6. General procedure for preparation of 5-Amino-3-phenylamino-1H-pyrazole-4-carboxylic acid amide (6) [22]

2-Cyano-3-methylsulfanyl-3-phenylamino-acrylamide (5) (8gm,

0.034 mol) was refluxed with methanol (250 ml) for  $1/2$  hr till completely dissolved then cooled. Hydrazine hydrate (1.6 ml, 0.034 mol) was added drop wisely on cold with continuous stirring for extra  $1/2$  hr, then followed with water bath for 3–4 hrs till precipitate occurred. The later was filtered and washed well with ether then crystallized from ethanol to give the title compound (6) as crystalline whitish grey powder (64%).

**6.1.6.1. 5-Amino-3-phenylamino-1H-pyrazole-4-carboxylic acid amide (6)**. Whitish grey powder; yield (64%); m.p.: 178–179 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3441 ( $\text{NH}_2$ ), 3379 ( $\text{NH}_2$ ), 3275 (NH), 3197 (NH), 1654 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 5.82 (s, 2H, Pyrazole- $\text{NH}_2$ , exchangeable by  $\text{D}_2\text{O}$ ), 6.74 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.17 (t, 1H, Ar-H), 7.2 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 7.33 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 8.97 (s, 1H, Pyrazole -NH, exchangeable by  $\text{D}_2\text{O}$ ), 11 (s, 2H, Amide- $\text{NH}_2$ , exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 217.2):  $m/z$ , 217.05 ( $\text{M}^+$ , [47.43%]).

#### 6.1.7. General procedure for preparation of 3-Carbamoyl-5-(4-substituted phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a]pyrimidine-6-carboxylic acid ethyl ester (7a-d) [23,24]

Hydrochloric acid (0.1 ml) was added to a mixture of aldehyde derivative (0.01 mol), ethyl acetoacetate (1.43gm, 0.01 mol) and 5-amino-3-phenyl-amino-1H-pyrazole-4-carboxylic acid amide (6) (2.17gm, 0.01 mol) in ethanol (40 ml) and then refluxed for 4–8 hrs. After that, the reaction mixture was poured upon ice and the resulting precipitate was filtered, washed by diethyl ether and crystallized from ethanol to give the titled compounds (7a-d).

**6.1.7.1. 3-Carbamoyl-5-(4-chloro-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (7a)**. Dark yellow powder; yield (80%); m.p.: 96–98 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3480 ( $\text{NH}_2$ ), 3325 (NH), 3093 (NH), 1720 (CO), 1658 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.5 (t, 3H,  $\text{CH}_3$ - $\text{CH}_2$ ), 2.73 (s, 3H, Pyrimidinyl- $\text{CH}_3$ ), 4.13 (dd, 2H,  $J = 8.6\text{Hz}$ ,  $\text{CH}_3$ - $\text{CH}_2$ ), 5.52 (s, 1H, Pyrimidinyl-H), 6.87 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.03 (t, 1H, Ar-H), 7.27 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 7.4 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 7.51 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 8.26 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.52 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ), 10 (s, 2H, Amide- $\text{NH}_2$ , exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 467.95):  $m/z$ , 467.1 ( $\text{M}^+$ , [1.03%]), 469.32 ( $\text{M}^{+2}$ , [0.91%]); **Anal.** Calcd., for  $\text{C}_{24}\text{H}_{26}\text{ClN}_5\text{O}_3$ ; C, 61.6; H, 5.6; N, 14.97; Found: C, 61.86; H, 5.89; N, 15.11.

**6.1.7.2. 3-Carbamoyl-5-(4-methoxy-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (7b)**. Grayish green powder; yield (81%); m.p.: 76–78 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3479 ( $\text{NH}_2$ ), 3332 (NH), 3271 (NH), 1708 (CO), 1658 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.5 (t, 3H,  $\text{CH}_3$ - $\text{CH}_2$ ), 2.72 (s, 3H, Pyrimidinyl- $\text{CH}_3$ ), 2.85 (s, 3H,  $\text{OCH}_3$ ), 4.14 (dd, 2H,  $J = 8.5\text{Hz}$ ,  $\text{CH}_3$ - $\text{CH}_2$ ), 5.47 (s, 1H, Pyrimidinyl-H), 6.87 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 6.97 (t, 1H, Ar-H), 7.13 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 7.44 (d, 2H,  $J = 8.4\text{Hz}$ , Ar-H), 7.73 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 8.17 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.55 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.71 (s, 2H, Amide- $\text{NH}_2$ , exchangeable by  $\text{D}_2\text{O}$ );  $^{13}\text{C NMR}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 15.5, 15.9, 55.59, 56.4, 60.1, 78.8, 110.66, 114.12, 114.56, 115.38, 115.9, 119.7, 128.5, 128.9, 129.6, 130.55, 135.32, 138.6, 148.6, 155.5, 156.9, 161.2, 164.5, 170.32; **MS**: (Mwt.: 447.49):  $m/z$ , 447.21 ( $\text{M}^+$ , [21.99%]); **Anal.** Calcd., for  $\text{C}_{24}\text{H}_{25}\text{N}_5\text{O}_4$ ; C, 64.42; H, 5.63; N, 15.65; Found: C, 64.63; H, 5.85; N, 15.84.

**6.1.7.3. 3-Carbamoyl-5-(4-hydroxy-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (7c)**. Off-white powder; yield (83%); m.p.: 90–92 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3425 (OH), 3309 ( $\text{NH}_2$ ), 3209 (NH), 2978 (NH), 1685 (CO), 1651 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.5 (t, 3H,  $\text{CH}_3$ - $\text{CH}_2$ ), 2.7 (s, 3H, Pyrimidinyl- $\text{CH}_3$ ), 4.02 (dd, overlapped 2H,  $\text{CH}_3$ - $\text{CH}_2$ ), 5.39 (s, 1H,

Pyrimidinyl-H), 6.66 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 6.86 (t, 1H, Ar-H), 7.12 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.42 (d, 2H,  $J = 8.4\text{Hz}$ , Ar-H), 7.84 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 8.22 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.39 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.58 (s, 1H, Ar-OH, exchangeable by  $\text{D}_2\text{O}$ ), 11.55 (s, 2H, Amide-NH<sub>2</sub>, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 433.46):  $m/z$ , 433.15 ( $\text{M}^+$ , [18.8%]); **Anal.** Calcd., for  $\text{C}_{23}\text{H}_{23}\text{N}_5\text{O}_4$ ; C, 63.73; H, 5.35; N, 16.16; Found: C, 63.98; H, 5.59; N, 16.34.

**6.1.7.4. 3-Carbamoyl-5-(4-fluoro-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (7d).** Grey powder; yield (80%); m.p.: 121–125 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3460 (NH<sub>2</sub>), 3336 (NH), 3217 (NH), 1697 (CO), 1662 (CO); **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 2.7 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>), 2.88 (s, 3H, Pyrimidinyl-CH<sub>3</sub>), 4.12 (dd, 2H, overlapped, CH<sub>3</sub>-CH<sub>2</sub>), 5.52 (s, 1H, Pyrimidinyl-H), 6.68 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 6.92 (t, 1H, Ar-H), 7.21 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.49 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.71 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 8.22 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.51 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.73 (s, 2H, Amide-NH<sub>2</sub>, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 435.45):  $m/z$ , 435.18 ( $\text{M}^+$ , [78.62%]); **Anal.** Calcd., for  $\text{C}_{23}\text{H}_{22}\text{FN}_5\text{O}_3$ ; C, 63.44; H, 5.09; N, 16.08; Found: C, 63.64; H, 5.21; N, 16.25.

#### 6.1.8. General procedure for preparation of 2-(Methylsulfanyl-phenylamino-methylene)-malononitrile (8) [20,21]

To a cooled solution of finely divided KOH (1.65gm, 0.025 mol), malononitrile (1.65gm, 0.025 mol) was added then followed by the addition of phenyl isothiocyanate (3.37gm, 0.025 mol). Reaction mixture was stirred at room temperature for 24 hrs then cooled again with addition of water (30 ml). Dimethyl sulphate (3.15gm, 0.025 mol) was added drop wisely then stirred continuously for extra 8 hrs to give a crystalline yellow precipitate. The precipitate was filtered and washed well with ether then crystallized from ethanol to give the title compound (8) (82%).

##### 6.1.8.1. 2-(Methylsulfanyl-phenylamino-methylene)-malononitrile (8)

Yellow powder; yield (82%); m.p.: 166–168 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3320 (NH), 2219 (CN), 2125 (CN); **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 3.9 (s, 3H, CH<sub>3</sub>), 7.28 (d, 2H,  $J = 8.4\text{Hz}$ , Ar-H), 7.4 (t, 1H,  $J = 8.5\text{Hz}$ , Ar-H), 7.53 (d, 2H, overlapped, Ar-H), 10.53 (s, 1H, NH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 215.27):  $m/z$ , 215.05 ( $\text{M}^+$ , [100%]).

#### 6.1.9. General procedure for preparation of 5-Amino-3-phenylamino-1H-pyrazole-4-carbonitrile (9) [22]

2-(Methylsulfanyl-phenylamino-methylene)-malononitrile (8) (7.3gm, 0.034 mol) was refluxed with methanol (250 ml) for  $\frac{1}{2}$  hr till completely dissolved then cooled. Hydrazine hydrate (1.6 ml, 0.034 mol) was added drop wisely on cold with continuous stirring for extra  $\frac{1}{2}$  hr, then followed with water bath for 3–4 hrs till precipitate occurred. The later was filtered and washed well with ether then crystallized from ethanol to give the title compound (9) as crystalline whitish grey powder as reported (80%).

**6.1.9.1. 5-Amino-3-phenylamino-1H-pyrazole-4-carbonitrile (9).** Whitish grey powder; yield (80%); m.p.: 204–205 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3468 (NH<sub>2</sub>), 3371 (NH), 3305 (NH), 2214 (CN), **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 6.23 (s, 2H, Pyrazole-NH<sub>2</sub>, exchangeable by  $\text{D}_2\text{O}$ ), 6.75 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.16 (t, 1H, Ar-H), 7.45 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 8.27 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 11.11 (s, 1H, Pyrazole-NH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 199.21):  $m/z$ , 199.09 ( $\text{M}^+$ , [100%]).

#### 6.1.10. General procedure for preparation of 5-(4-substituted-phenyl)-3-cyano-7-methyl-2-phenylamino-4,5-dihydro-pyrazolo[1,5-a]pyrimidine-6-carboxylic acid ethyl ester (10a-e) [23,24]

Hydrochloric acid (0.1 ml) was added to a mixture of aldehyde

derivative (0.01 mol), ethyl acetoacetate (1.43gm, 0.01 mol) and 5-amino-3-phenylamino-1H-pyrazole-4-carbonitrile (9) (1.99gm, 0.01 mol) in ethanol (40 ml) and then refluxed for 4–8 hrs. After that, the reaction mixture was poured onto ice and the resulting precipitate was filtered, washed well by diethyl ether and crystallized from ethanol to give the titled compounds (10a-e).

**6.1.10.1. 5-(4-Chloro-phenyl)-3-cyano-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (10a).** Dark yellow powder; yield (81%); m.p.: 177–179 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3421 (NH), 3317 (NH), 2210 (CN), 1685 (CO); **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 2.69 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>), 2.83 (s, 3H, Pyrimidinyl-CH<sub>3</sub>), 4.12 (dd, 2H, overlapped, CH<sub>3</sub>-CH<sub>2</sub>), 5.48 (s, 1H, Pyrimidinyl-H), 6.88 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.24 (t, 1H, Ar-H), 7.34 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.63 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.76 (d, 2H, overlapped, Ar-H), 8.91 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.15 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 433.89):  $m/z$ , 433.13 ( $\text{M}^+$ , [100%]), 435.12 ( $\text{M}^{+2}$ , [29.27%]); **Anal.** Calcd., for  $\text{C}_{23}\text{H}_{20}\text{ClN}_5\text{O}_2$ ; C, 63.67; H, 4.65; N, 16.14; Found: C, 63.91; H, 4.86; N, 16.35.

**6.1.10.2. 3-Cyano-5-(4-methoxy-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (10b).** Pale green powder; yield (82%); m.p.: 178–180 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3363 (NH), 3325 (NH), 2218 (CN), 1674 (CO); **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 2.48 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>), 2.72 (s, 3H, Pyrimidinyl-CH<sub>3</sub>), 3.68 (s, 3H, OCH<sub>3</sub>), 4.07 (dd, 2H,  $J = 8.5\text{Hz}$ , CH<sub>3</sub>-CH<sub>2</sub>), 5.4 (s, 1H, Pyrimidinyl-H), 6.84 (d, 2H, overlapped, Ar-H), 6.88 (t, 1H, Ar-H), 7.14 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.22 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.59 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 8.87 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.05 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ); **<sup>13</sup>C NMR** (75 MHz, DMSO- $d_6$ )  $\delta$ : 16.5, 16.9, 55.6, 56.5, 60.2, 75.8, 108.66, 113.12, 113.56, 115.5, 115.9, 118, 118.8, 128.25, 128.7, 129.66, 131.58, 134.12, 139.76, 150.32, 154.5, 155.9, 161.2, 164.5; **MS**: (Mwt.: 429.47):  $m/z$ , 429.21 ( $\text{M}^+$ , [100%]); **Anal.** Calcd., for  $\text{C}_{24}\text{H}_{23}\text{N}_5\text{O}_3$ ; C, 67.12; H, 5.40; N, 16.31; Found: C, 67.31; H, 5.69; N, 16.53.

**6.1.10.3. 3-Cyano-5-(4-hydroxy-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (10c).** Light brown powder; yield (80%); m.p.: 240–242 °C; (CO); **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3429 (OH), 3379 (NH), 3286 (NH), 2202 (CN), 1708 (CO); **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 2.58 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>), 3.22 (s, 3H, Pyrimidinyl-CH<sub>3</sub>), 4.07 (dd, 2H, overlapped, CH<sub>3</sub>-CH<sub>2</sub>), 5.36 (s, 1H, Pyrimidinyl-H), 6.72 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 6.86 (t, 1H, Ar-H), 7.04 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.23 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.62 (d, 2H, overlapped, Ar-H), 8.9 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.45 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ), 13 (s, 1H, Ar-OH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 415.44):  $m/z$ , 415.16 ( $\text{M}^+$ , [100%]); **Anal.** Calcd., for  $\text{C}_{23}\text{H}_{21}\text{N}_5\text{O}_3$ ; C, 66.49; H, 5.09; N, 16.86; Found: C, 66.72; H, 5.21; N, 17.03.

**6.1.10.4. 3-Cyano-5-(4-fluoro-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a] pyrimidine-6-carboxylic acid ethyl ester (10d).** Grey powder; yield (83%); m.p.: > 300 °C; **IR**: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3410 (NH), 3271 (NH), 2214 (CN), 1678 (CO); **<sup>1</sup>H NMR** (300 MHz) (DMSO- $d_6$ )  $\delta$ : 2.47 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>), 3.28 (s, 3H, Pyrimidinyl-CH<sub>3</sub>), 4.07 (dd, 2H, overlapped, CH<sub>3</sub>-CH<sub>2</sub>), 5.46 (s, 1H, Pyrimidinyl-H), 6.87 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.17 (t, 1H, Ar-H), 7.24 (d, 2H,  $J = 8.7\text{Hz}$ , Ar-H), 7.28 (d, 2H,  $J = 8.5\text{Hz}$ , Ar-H), 7.6 (d, 2H,  $J = 8.6\text{Hz}$ , Ar-H), 8.88 (s, 1H, Ar-NH, exchangeable by  $\text{D}_2\text{O}$ ), 9.11 (s, 1H, Pyrimidinyl-NH, exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 417.44):  $m/z$ , 417.16 ( $\text{M}^+$ , [100%]); **Anal.** Calcd., for  $\text{C}_{23}\text{H}_{20}\text{FN}_5\text{O}_2$ ; C, 66.18; H, 4.83; N, 16.78; Found: C, 66.17; H, 4.99; N, 16.91.

**6.1.10.5. 3-Cyano-5-(4-dimethylamino-phenyl)-7-methyl-2-phenylamino-4,5-dihydro pyrazolo[1,5-a]pyrimidine-6-carboxylic acid ethyl ester**

**(10e)**. Dark orange powder; yield (81%); m.p.: 89–91 °C; IR: ( $\nu$  max,  $\text{cm}^{-1}$ ): 3325 (NH), 3151 (NH), 2210 (CN), 1651 (CO);  $^1\text{H NMR}$  (300 MHz)(DMSO- $d_6$ )  $\delta$ : 2.47 (t, 3H,  $\text{CH}_3\text{-CH}_2$ ), 2.82 (s, 6H, N( $\text{CH}_3$ ) $_2$ ), 3.3 (s, 3H, Pyrimidinyl- $\text{CH}_3$ ), 4.06 (dd, overlapped, 2H,  $\text{CH}_3\text{-CH}_2$ ), 5.33 (s, 1H, Pyrimidinyl- $\text{H}$ ), 6.62 (d, 2H,  $J = 8.6\text{Hz}$ , Ar- $\text{H}$ ), 6.85(d, 2H,  $J = 8.5\text{Hz}$ , Ar- $\text{H}$ ), 7.16 (t, 1H, Ar- $\text{H}$ ), 7.24 (d, 2H,  $J = 8.7\text{Hz}$ , Ar- $\text{H}$ ), 7.64 (d, 2H,  $J = 8.7\text{Hz}$ , Ar- $\text{H}$ ), 8.87 (s, 1H, Ar- $\text{NH}$ , exchangeable by  $\text{D}_2\text{O}$ ), 8.99 (s, 1H, Pyrimidinyl- $\text{NH}$ , exchangeable by  $\text{D}_2\text{O}$ ); **MS**: (Mwt.: 442.51):  $m/z$ , 442.23 ( $\text{M}^+$ , [3.84%]); **Anal.** Calcd., for  $\text{C}_{25}\text{H}_{26}\text{N}_6\text{O}_2$ ; C, 67.86; H, 5.92; N, 18.99; Found: C, 69.11; H, 6.21; N, 19.23.

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## Appendix A. Supplementary material

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