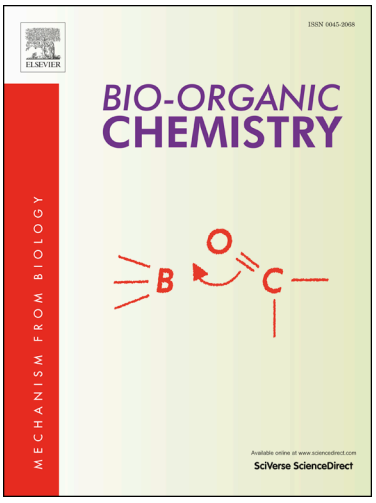


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# Design and synthesis of new phthalazine-based derivatives as potential EGFR inhibitors for the treatment of hepatocellular carcinoma

**Ahmed T. A. Boraei[a*], Hanaa K. Ashour[a], El Sayed H. El Tamany[a], Nahla Abdelmoaty[a], Abdullah I. El-Falouji[b], Mohamed S. Gomaa[c]**

[a]*Chemistry Department, Faculty of Science, Suez Canal University, Ismailia, Egypt*

[b]*Institute of Biotechnology for Postgraduate Studies and Research, Suez Canal University, Ismailia, Egypt*

[c]*Chemistry Department, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam, Kingdom of Saudi Arabia*

## Abstract:

Searching for new leads in the battle of cancer will never ends, we herein disclose the design and synthesis of new phthalazine derivatives and their *in vitro* and *in vivo* testing for their antiproliferative activity. Phthalazine was selected as a privilege moiety that is incorporated in a big number of anticancer drugs in clinical use or that are still under clinical or preclinical studies. We utilized the drug extension strategy to tailor the designed compounds to fit the EGFR hydrophobic sub pocket and cleft region. The designed phthalazine derivatives was synthesized by linking phthalazine moiety with 1,3,4-oxadiazole-thione and 1,2,4-triazole-thione. Alkylation and glycosylation of the new heterocyclic systems were successfully performed to be used in the drug extension. Coupling

of some phthalazine derivatives with different amino acids was also performed to improve the drug selectivity.

The synthesized compounds were tested for their antiproliferative activity against cancer cells both *in vivo* and *in vitro*. The *in vitro* activity against hepatocellular carcinoma (HepG2 cell line) ranged from 5.7 µg/mL to 43.4 µg/mL. Compounds **31a** and **16** were the most active with an $IC_{50}$ 5.7 µg/mL and 7.1 µg/mL, respectively compared to the standard compound doxorubicin (4.0 µg/mL). *In vivo*, compounds **10** and **16** showed $IC_{50}$ values 7.25 µM and 7.5 µM, respectively compared to the standard compound cisplatin ($IC_{50}$ 9.0 µM). *In silico*, testing of the phthalazine derivatives showed that they are good inhibitors for EGFR. The docking studies substantiated compounds **4**, **10**, **16** and **31a** as new lead compounds and identified Arg841 as a key residue in the cleft region for binding stronger inhibitors.

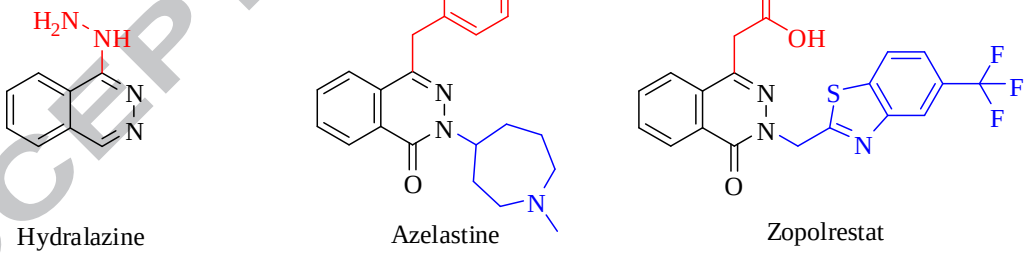
**Corresponding Author Email Address:** Ahmed_Tawfeek83@yahoo.com

**Keywords:** Anticancer activity; HepG2; phthalazines; alkylation; 1,3,4-oxadiazolethione; 1,2,4-triazolethione

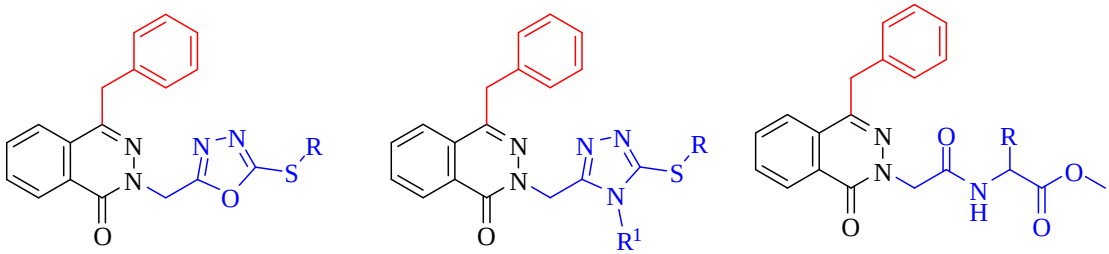
## 1. Introduction

Cancer is a main public health problem worldwide and is the second leading cause of death in the United States.The continuous decline in cancer death rates over the last 2 decades is due to the continuous effort of researchers to overcome this disease [1].

It is well known that phthalazinone derivatives have great interest due to their antidiabetic [2], vasorelaxant [3], antiallergic [4], PDE4 inhibitors [5], vascular endothelical group factor receptor (VEGFR) tyrosine kinase inhibitors for treatment of cancer [6,7], antiasthmatic [8] and herbicidal agents [9]. Drug molecules like hydralazine [10,11], burdralazine [12,13], ponalrest [14], azelastine [15,16], and zopolrestat [17]. Several phthalazine derivatives were found to have antitumor [18-20], anticovulsant [21,22], antihypertensive [23,24], antitrypanosomal [25], antimicrobial [26], and anti-inflammatory activities [27,28]. Since, most of drugs containing phthalazines are substituted at position 2 and 4 of phathalazine scaffold; the present study is concerned with changing the substituents at those two positions to find potent anticancer leads (Fig. 1.




**Selected drugs**



**Planned structures**

**Fig. 1.** Structures of selected phthalazine containing drugs and planned structures.

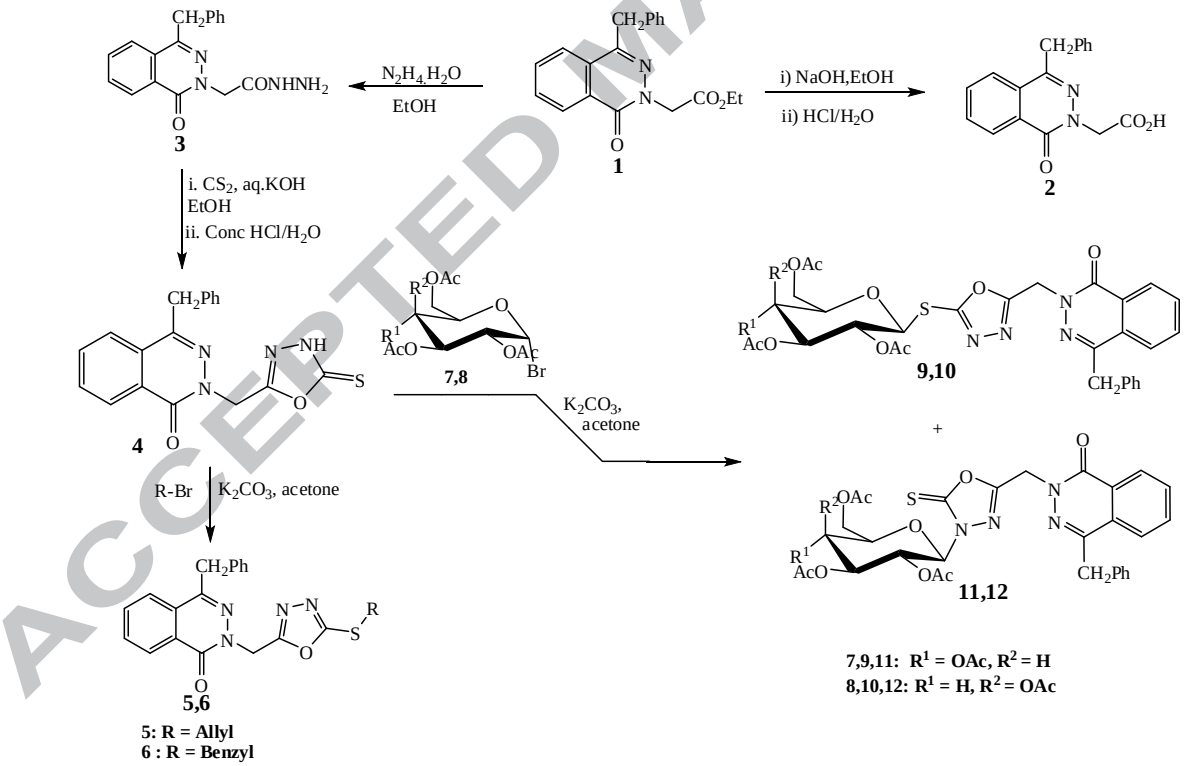
In continuation to our previous work [29], on the development of new tyrosine kinase inhibitors for the treatment of cancer, which resulted on a new indolyl-triazole lead, we hereby present the development of new lead inhibitors for EGFR. The presence of more than one compound to choose from would make future development and optimization easier in term of pharmacokinetic properties and toxicity profile optimization. These promising lead compounds will be further optimized or even hybridized and tested in a hope to reach a final compound that would progress to clinical trials and development. We designed compounds that would form better interactions with residues at the active site than our previous lead and have variable log P and pKa. We utilized ring variation, substituent variation, chain extension, introduction of hydrogen-donors and hydrogen acceptors techniques in our new lead search.

## 2. Results and discussion

### 2.1. Chemistry

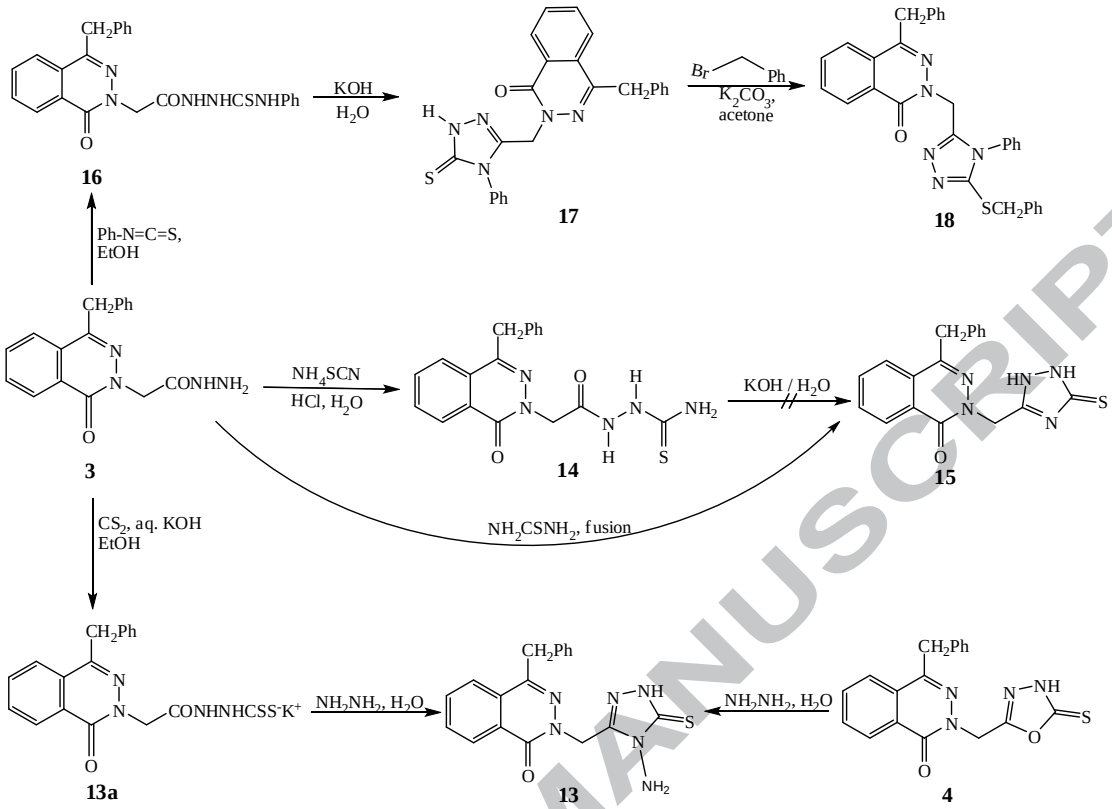
2-(4-Benzyl-1-oxophthalazin-2(1*H*)-yl)acetic acid **2** was obtained from the hydrolysis of ethyl 2-(4-benzyl-1-oxophthalazin-2(1*H*)-yl)acetate **1** in alcoholic sodium hydroxide whereas, reaction of ester **1** with hydrazine hydrate in ethanol afforded hydrazide **3**. Refluxing hydrazide **3** with

carbon disulphide in ethanol containing aq. KOH then acidification led to the formation of 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazol-2-thione **4**. Alkylation of oxadiazole **4** with allyl bromide and benzyl bromide in the presence of potassium carbonate afforded *S*-allyl-1,3,4-oxadiazole **5** and *S*-benzyl-1,3,4-oxadiazole **6** respectively. Whereas, glycosylation of the oxadiazole **4** with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **7** and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **8** yielded a mixture of *S*-glycosylated **9**,**10** and *N*-glycosylated analogs **11**,**12** (Scheme 1).




**Scheme 1:** Synthesis, alkylation, and glycosylation of phthalazino-1,3,4-oxadiazolethione **4**.

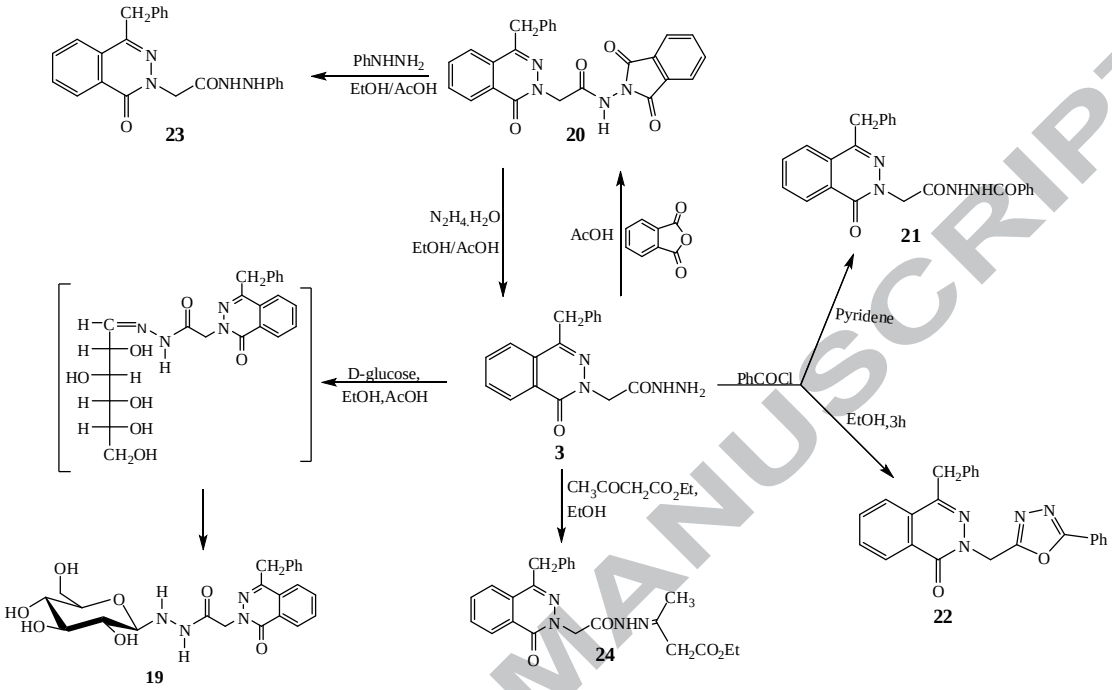
Stirring the hydrazide **3** with carbon disulphide in ethanol containing aq. KOH gave potassium dithiocarbazate **13a** which was separated and then refluxed with hydrazine hydrate in water to afford 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-4-amino-1,2,4-triazole-3-thione **13** after cooling and acidification. Reaction of hydrazide **3** with ammonium thiocyanate in water containing hydrochloric acid led to the formation of 1-((4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetyl)thiosemicarbazide **14** which failed to cyclize to dihydro-1,2,4-triazole **15** upon treating with aq. KOH and acidification. While, dihydro-1,2,4-triazole **15** was successfully obtained from fusion of hydrazide **3** with thiourea. Stirring of hydrazide **3** with phenyl isothiocyanate in absolute ethanol led to the formation of 1-((4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetyl)-4-phenyl-thiosemicarbazide **16**. Cyclization of **16** was done by reflux in aq. KOH to the form 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-4-phenyl-1,2,4-triazol-3-thione **17**. Benzylation of 1,2,4-triazole **17** with benzyl bromide in existence of potassium carbonate afforded *S*-benzyl-4-phenyl-1,2,4-triazole **18** (Scheme 2).




**Scheme 2:** Synthesis of diverse 1,2,4-triazolo-phthalazines.

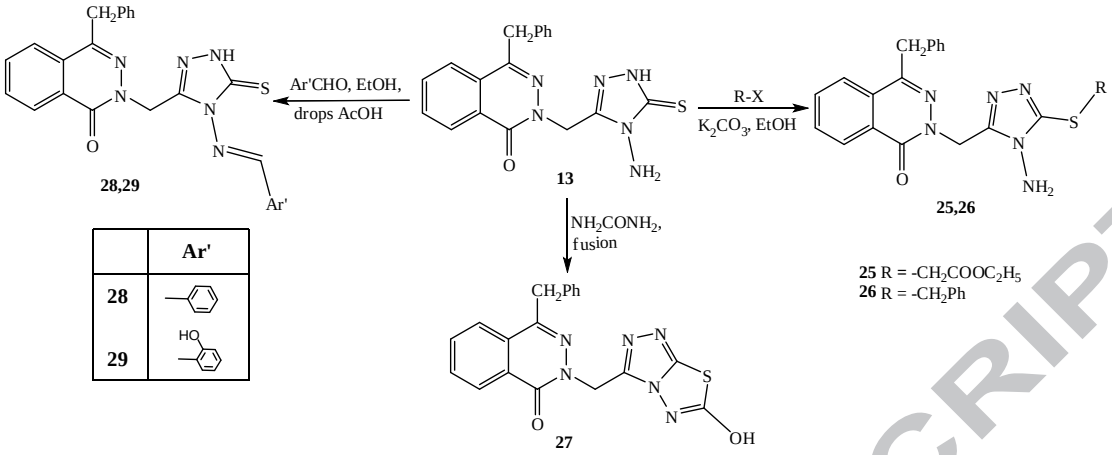
In addition, refluxing hydrazide **3** with α-D-glucose in ethanol containing few drops of glacial acetic acid yielded 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N*'-(*β*-D-glucopyranosyl)acetohydrazide **19**. While, reaction of **3** with phthalic anhydride under the same conditions led to the formation of 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N*-(1,3-dioxoisoindolin-2-yl)acetamide **20**. Benzoylation of hydrazide **3** with benzoyl chloride was highly dependent on the media used. The use of pyridene yielded *N*'-(2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetyl)benzohydrazide **21**, whereas, when done in ethanol gave 2-phenyl-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazole **22**. Treatment of **20** with phenyl hydrazine in ethanol containing few drops of glacial acetic acid led to the formation of *N*'-phenylacetohydrazide **23**. Similarly hydrazinolysis of **20** at the same conditions afforded the hydrazide **3**. The reaction of hydrazide **3** with ethyl acetoacetate in ethanol led to the formation of ethyl 3-(2-(2-(4-

benzyl-1-oxo-1*H*-phthalazin-2-yl)acetyl)hydrazono)butanoate **24**. (Scheme 3).




**Scheme 3:** Reactions of hydrazide **3** with D-glucose, phthalic anhydride, benzoyl chloride, ethylacetoacetate and phenyl hydrazine.

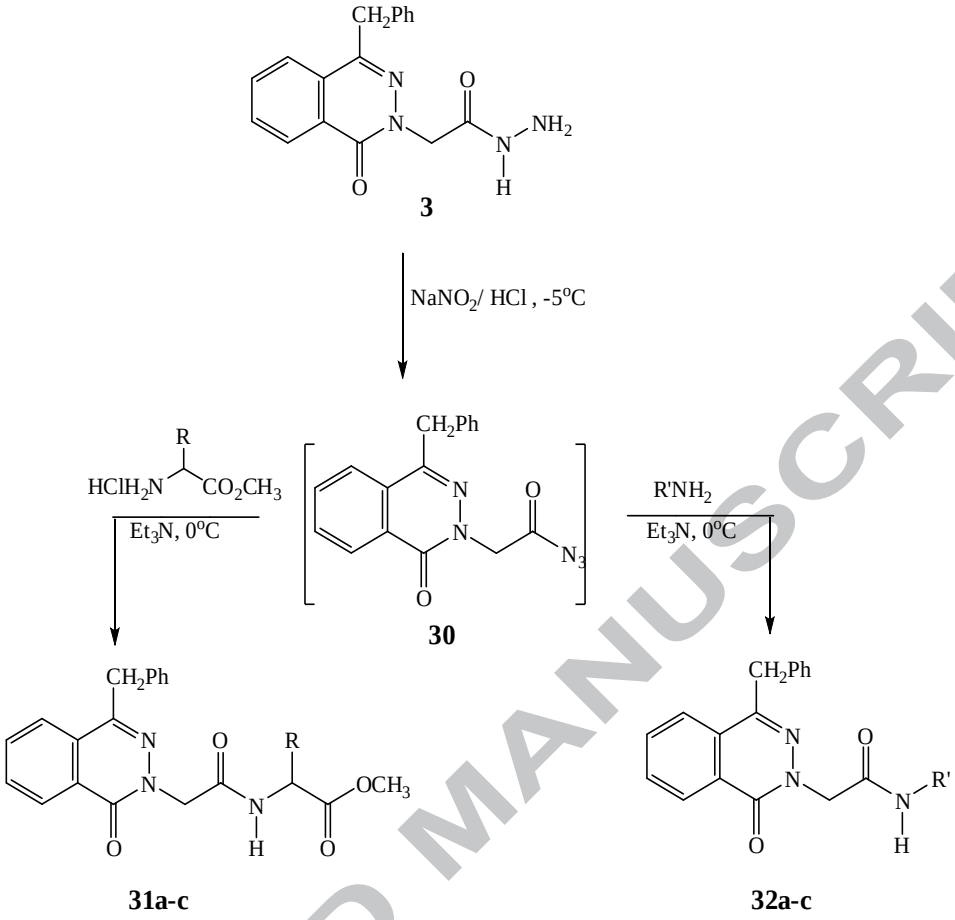
Alkylation of 1,2,4-triazole **13** with ethyl chloroacetate and benzyl chloride in the existence of potassium carbonate afforded *S*-alkylated-4-amino-1,2,4-triazole **25** and **26** respectively. Triazolo-thiazole **27** was obtained by fusion of 1,2,4-triazole **13** and urea. Condensation of 4-amino-1,2,4-triazole **13** with aromatic aldehydes namely benzaldehyde and salicylaldehyde in ethanol containing acetic acid afforded the formation of 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-4-(arylideneamino)-1,2,4-triazole-3-thione **28** and **29** respectively (Scheme 4).



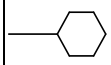

| | Ar' |
|---|---|
| 28 | |
| 29 | |

**Scheme 4:** Alkylation and condensation of 4-amino-1,2,4-triazole **13.**

Reaction of hydrazide **3** with $NaNO_2$/HCl at low temperature yielded the azide **30** which was coupled with a number of amino acids esters (glycine, L-leucine and L-serine) in the presence of triethyl amine to give coupled products **31a-c** respectively. Azide-coupling method was effectively applied for coupling of azide **30** with benzyl amine, cyclohexylamine and n-propylamine to produce the amides **32a-c** (Scheme 5).




| 31 | R |
|---|---|
| **a** (Gly) | -H |
| **b** ( leu) | $-CH_2CH(CH_3)_2$ |
| **c** (ser) | $-CH_2OH$ |

| 32 | R' |
|---|---|
| **a** (benzyl) | $-CH_2Ph$ |
| **b** ( cyclohexyl) |  |
| **c** (propyl) | $-CH_2CH_2CH_3$ |

**Scheme (5)**: Coupling of azide **30** with amino acid esters and amines.

*Significant analytical data used for structures confirmation*

The $^{1}$H-NMR spectra of all compounds displayed a sharp singlet signal around 4.30 ppm for -C*H*$_2$Ph directly attached to C-4 of phthalazine ring. The methylene protons attached to N-2 of phthalazine (*N*-C*H*$_2$) appeared around 4.80 ppm as in **2** and **3** which revealed deshielding as a result of the cyclization to appear around 5.60 ppm as in the rest of the compounds. The aromatic protons appeared between 7.18-8.30 ppm. $^{13}$C-

NMR spectra revealed the methylene carbon of the -$CH_2$Ph group (attached to C-4) at 37.5ppm. While, N$CH_2$ (attached to N-2) appeared between 45.0 and 52.0 ppm. All the aromatic carbons were found between 125.7 and 146.0 ppm. The carbonyl group of the phthalazinone ring was observed around 158.0 ppm. The additional significant data could be discussed as following:

The $^1$H-NMR spectrum of the acid **2** showed the carboxylic acid OH signal$_{(broad)}$ at 13.04 ppm and the respective carbonyl group of the acid appeared at 169.4 ppm. The NMRs of the hydrazide **3** showed the hydarzino (N*H*N$H_2$) group protons at 4.30 and 9.31 ppm and the carbonyl group appeared at 166.4 ppm.

$^1$H-NMR spectrum of oxadiazolethione **4** revealed abroad signal at 14.60 ppm for the NH of the oxadiazolethione. $^{13}$C-NMR spectrum showed the two oxadiazole signals (C-5$_{Oxad.}$) at 154.8 and (C=S$_{Oxad.}$) at169.4 ppm.

Due to the allylation of oxadiazole **4**, $^1$H-NMR spectrum of compound **5** showed S$CH_2$ signal at 3.87. The olefinic methylene protons (CH=C$\underline{H_2}$) appeared as two doublet of doublets at 5.07 ppm for the cis proton with coupling constant values $J_{Cis}$ 9.0, $J_{Gem}$ 0.9 Hz, while the trans proton appeared at 5.26 ppm with coupling constant $J_{Trans} \approx 16$, $J_{Gem}$ 1.2 Hz. The olefinic CH (C$\underline{H}$=$CH_2$) appeared as multiplet at 5.92-5.98 ppm. $^{13}$C-NMR spectrum showed a signal at 34.5 ppm corresponding to (S$CH_2$). The NMR spectrum of compound **6** showed a singlet signal for benzyl methylene protons attached to sulfur (SC$\underline{H_2}$Ph) at 4.46 ppm and the corresponding methylene carbon at 35.8 ppm. $^1$H- and $^{13}$C-NMRs unambiguously differentiated between *S*- and *N*-glycosides as following: *S*-glycosides **9** and **10** showed the anomeric protons around 5.66 ppm. Whereas, *N*-glycosides **11** and **12** displayed their anomeric protons around 6.20. All anomeric protons revealed large coupling constant value $J_{1,2} > 9.3$ Hz which confirm the $\beta$-configuration. Moreover, $^{13}$C-NMR of

glycosides **11** and **12** displayed C=S signal at 177.3 ppm which supports the assignment. The structure of 4-amino-1,2,4-triazole **13** was confirmed from $^{1}$H-NMR which showed two singlet signals at 5.63 ppm and 13.67 ppm which corresponding to $NH_2$ and NH respectively. $^{13}$C-NMR showed the two triazole carbon signals at 148.1 and 166.3 ppm due to C-$5_{Triazol}$ and C=S respectively.

The structure of the thiosemicarbazide **14** was confirmed from $^{1}$H-NMR which showed two singlet signals at 9.44 and 10.26 ppm for the two NH groups (CONH-NH-). $^{13}$C-NMR spectrum revealed two signals at 166.6 and 182.0 ppm corresponding to carbonyl of amide and thiocarbonyl (C=S) groups. $^{1}$H-NMR spectrum of dihydro-1,2,4-triazole-3-thione **15** showed two singlet signals at 13.38 and 13.41 ppm due to two NH of triazole ring. $^{13}$C-NMR spectrum presented two triazole carbon signals at 148.8 and 166.6 ppm.

The phthalazino-phenyl thiosemicarbazide **16** structure was elucidated from $^{1}$H-NMR which displayed three NH signals (CON*H*N*H*CS-N*H*Ph) at 9.60, 9.81 and 10.46 ppm. $^{13}$C-NMR spectrum revealed two signals due to C=O and (C=S) at 166.9 and 181.0 ppm respectively. $^{1}$H-NMR spectrum of phthalazino-4-phenyl-1,2,4-triazole-3-thione **17** showed a singlet signal at 13.91 ppm for NH group. $^{13}$C-NMR spectrum revealed the two triazole carbon signals 148.3 ppm (C=$N_{Triazol}$) and 168.1 ppm (C=S). The NMRs spectra of *S*-benzyl-1,2,4-triazole **18** showed a singlet signal for benzyl methylene protons attached to sulfur (*SCH*$_2$Ph) at 4.19 ppm and the corresponding methylene carbon appeared at 36.2 ppm. $^{1}$H-NMR of the glucoside **19** (in DMSO-$d_6$ + $D_2O$) showed multiplet signal between 3.01-3.77 ppm corresponding to glucose six protons (H-$2_{Glu}$, H-$3_{Glu}$, H-$4_{Glu,}$ H-$5_{Glu,}$ H-$6_{Glu}$ and H-6´$_{Glu}$). The anomeric proton appeared as doublet at 3.85 ppm with coupling constant $J_{2,3}$ 9.0 Hz. A doublet signal was appeared between 5.04-5.29 ppm with coupling constant value

$J_{Gem}$17.1 Hz corresponding to $NCH_2$ group. $^{13}$C-NMR spectrum showed the six glucose signals at 64.3, 64.9, 73.2, 73.8, 79.5 and 94.0 ppm. $^{1}$H-NMR spectrum of compound **20** revealed a singlet signal at 11.08 ppm due to NH group. $^{13}$C-NMR spectrum showed two signals at 164.8 and 166.8 ppm corresponding to three carbonyl carbons. $^{1}$H-NMR spectrum of compound **21** showed two singlet signals at 10.31 and 10.48 ppm corresponding to two NHs of (-CO-NHNHCOPh). $^{13}$C-NMR showed two signals at 165.3 and 166.4 ppm for the two carbonyl groups. The structure of 2-phenyl-1,3,4-oxadiazole **22** was deduced from $^{1}$H-NMR which displayed $NCH_2$ signal at 4.96 ppm and the corresponding carbon at 52.4 ppm.

$^{1}$H-NMR spectrum of the phenylacetohydrazide **23** showed the two NH signals (-CONHNHPh) at 2.87 and 10.37 ppm. $^{13}$C-NMR revealed a signal at 165.9 corresponding to carbonyl of amide group. $^{1}$H-NMR of compound **24** displayed the two $CH_3$ at 1.21 ppm and 1.97 ppm. The methylene protons appeared as singlet at 3.37 ppm and quartet at 4.14 ppm. The NH signal appeared at 10.72 ppm. $^{13}$C-NMR spectrum revealed three methylene carbons at 44.5, 52.8 and 60.9 ppm.

The structure of **25** was deduced from $^{1}$H-NMR which demonstrated triplet and quartet signals at 1.17 and 4.09 ppm corresponding to the methyl and methylene protons of the ethoxy group ($OCH_2CH_3$). $^{13}$C-NMR revealed the ethoxy carbons ($OCH_2CH_3$) signals at 14.4 and 61.6 ppm. The carbonyl of the ester group was appeared at 168.9 ppm. $^{1}$H-NMR of compound **26** showed the methylene protons of the new attached benzyl group as singlet at 4.39 ppm and the corresponding carbon was found at 34.7 ppm. NMRs of **27** displayed the spacer methylene protons at 5.44 ppm and the corresponding methylene carbon at 44.8 ppm in addition to a broad signal for hydroxyl group at 13.62 ppm. The structure of **28** was elucidated from $^{1}$H-NMR which showed the disappearance of

$NH_2$ signal and instead, a singlet signal was appeared 9.94 for benzylidene group (N=C*H*). $^{13}$C-NMR revealed signal at 162.6 ppm for C=S. The structure of **29** was elucidated from $^{1}$H-NMR which displayed three singlet signals at 9.00, 10.29 and 13.94 ppm for benzylidene N=C*H*, hydroxyl and NH groups respectively. $^{13}$C-NMR spectrum showed C=S signal at 162.7 ppm.

The structures of the coupled amino acid esters were deduced from their NMR spectra which demonstrated the methoxy protons ($OCH_3$) as singlet at 3.66 ppm while the corresponding carbon appeared around 52.2 ppm. In addition, two signals appeared in $^{13}$C-NMR around 168.0 and 173.2 ppm for the carbonyl of ester and amide. Moreover, the characteristic amino acid signals could be discussed as following:

$^{1}$H-NMR of Glycinated phthalizinone **31a** showed the glycine C$H_2$ as singlet at 3.93 ppm and glycine NH at 8.55 ppm. $^{13}$C-NMRspectrum revealed a signal at 41.1 attributable to glycine $CH_2$. $^{1}$H-NMR spectrum of Leucinated phthalizinone **31b** revealed leucine methyl protons at 0.90 ppm, CH and $CH_2$ were observed around 1.58 and 1.70 ppm. $^{13}$C-NMR spectrum revealed four signals at 21.9, 23.1, 24.7 and 50.9 ppm corresponding to $2CH_3$, CH, $CH_2$ and NCH groups of leucine respectively. $^{1}$H-NMR spectrum of serinated phthalizinone **31c** showed serine OH at 4.73 ppm, $CH_2$ at 3.67 ppm and CH at 4.46 ppm. $^{13}$C-NMR showed two signals at 55.3 and 61.9 ppm for N*C*H and *C*$H_2$OH. NMR of compound **32a** showed the methylene protons of the benzyl amine at 4.36 and the respective methylene carbon at 42.7 ppm. While, the NH of amine appeared at 8.64 ppm. NMR spectrum of **32b** revealed a multiplet signal at 1.10-1.83 ppm corresponding to five methylene groups of the cyclohexyl moiety whereas, the remaining CH was observed at 3.59 ppm. The carbons of the cyclohexyl moiety appeared at 25.0, 25.7, 32.9 and 48.3 ppm. $^{1}$H-NMR spectrum of **32c** displayed the propyl signals at 0.87,

1.41, 3.11 ppm. $^{13}$C-NMRshowed the propyl signals at 11.8 ($CH_3$), 22.8 ($CH_2$) and 53.6 ppm ($NCH_2$).

## 2.2. Anticancer Activity

The newly synthesized compounds were test for their anticancer activity in *vitro* against human hepatocellular carcinoma cell line HepG2 with comparison to the reference anticancer drug doxorubicin.

The method of Skehan et al., [30] was used to test the potential cytotoxicity of the compounds. Different concentrations of the compound under test (0, 5, 12.5, 25 and 50 µg/mL) were added to the cell line. After 48 hr of incubation at 37$^{o}$C and in atmosphere of 5% $CO_2$, cells were fixed, washed and stained with Sulfo-Rhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered withTris EDTA buffer and color intensity was measured in an ELISA reader. The relation between surviving fraction and compound concentration is plotted to get the survival curve of tumor cell line after the specified compound and determine the $IC_{50}$ of the compound.

### 2.2.1. In *vitro* Anti-Tumor results

The synthesized phthalazine derivatives were biologically evaluated for their antiproliferative activity against HepG2 cell lines where they showed promising activity (Table 1). The activity ranged from 5.7-43.4 µg/mL. Compounds **31a** and **16** were the most active with an $IC_{50}$ of 5.7 µg/mL and 7.1 µg/mL respectively compared to the standard compound doxorubicin (4.0 µg/mL). Compounds **4**, **10** and **13** showed activity in the tenth micromolar range; 15.8 µg/mL, 13.6 µg/mL, 16.2 µg/mL respectively. All the remaining compounds exhibited lower but comparable activity. The obtained biological results make this new

phthalazine series a good catch for further development and testing. Our future directions will focus on biomedical assays to substantiate the claimed activity against EGFR together with synthesizing more derivatives for testing.

**Table( 1 )** $IC_{50}$ and binding affinities of the docked compounds in EGFR calculated with autodock 4.3.

| Compd. Code | $IC_{50}$ (µg/mL) | Binding Affinity (kcal/mol) |
|---|---|---|
| **Doxorubicin** | 4.0 | |
| **4** | 15.8 | -9.6 |
| **10** | 13.6 | -8.6 |
| **12** | 28 | -8.1 |
| **13** | 16.2 | -9.8 |
| **14** | 37.9 | -9.2 |
| **16** | 7.09 | -10.8 |
| **17** | 39.2 | -9.7 |
| **18** | 43.4 | -10.2 |
| **24** | 37.3 | -9.7 |
| **26** | 24.3 | -11.0 |
| **27** | 20.3 | -10.3 |
| **28** | 29.3 | -10.2 |
| **29** | 36 | -11.1 |
| **31a** | 5.7 | -9.3 |
| **31b** | 29.3 | -8.9 |
| **31c** | 43.4 | -8.8 |
| **32a** | 41.4 | -9.6 |
| **32c** | 31.3 | -9.1 |

Figures **(2** and **3)** shows a dramatic decrease in tumor cell viability of human liver carcinoma cell line HepG2 following 48 hr treatments. Increasing the concentration of compounds **10** and **16** from 0.0 mM to 50 mM resulted in an increase in the percentage of cell inhibition as seen in the figures. The results of the cell viability tests imply that these compounds can be used to arrest the proliferation of tumor cells.

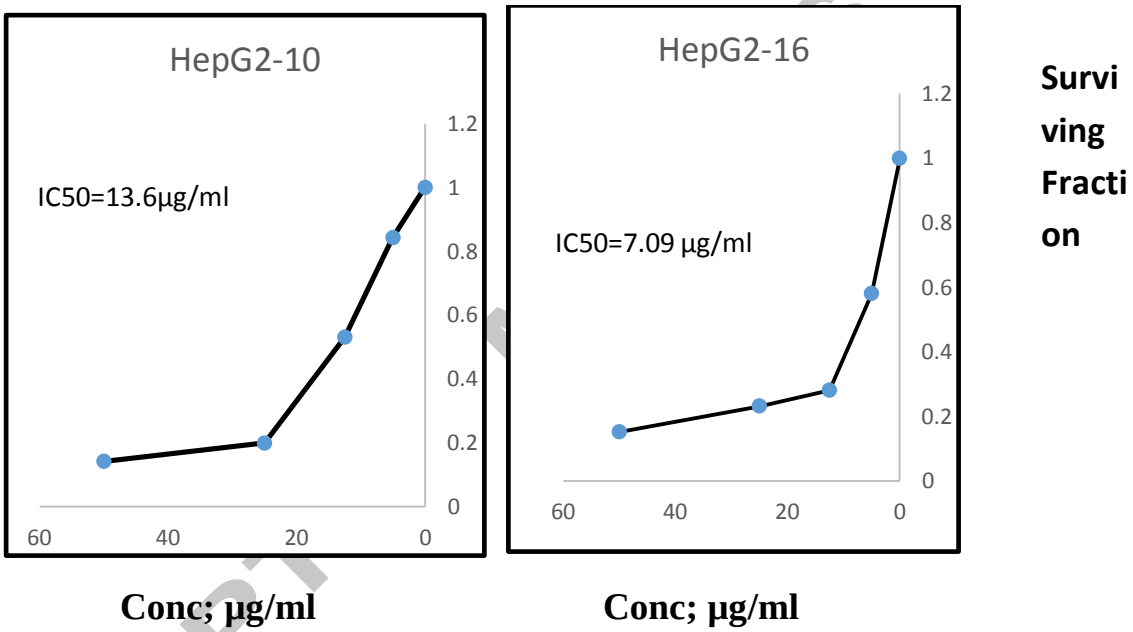



**Fig. 2** and **3.**The cytotoxic effect of compounds **10** and **16** on HepG2 data represent mean ± S.E.

The antiproliferative activities of the tested compounds were comparable to doxorubicin. The most active compounds were **4**, **10**, **16**, and **31a** which give $IC_{50}$ values 15.8 µM, 13.6 µM, 7.09 µM and 5.7 µM respectively (Fig. 4, Table 1).

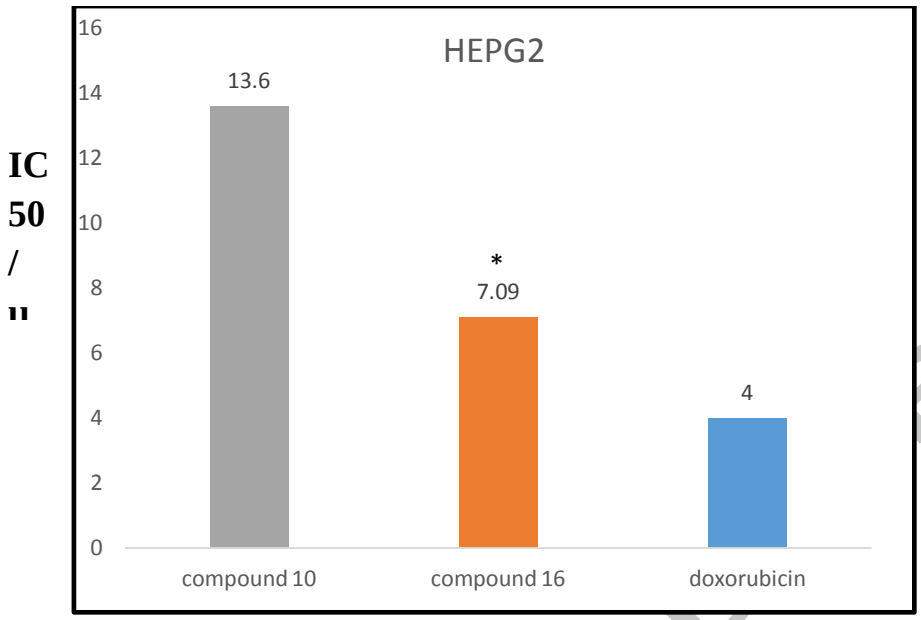



**Fig. 4**. **I**$IC_{50}$ values for compounds **10** and **16** against HepG2 (μM). $IC_{50}$ values are presented as the mean ± SD (standard error of the mean) obtained from three independent experiments.

### 2.2.2. In *vivo* Anti-Tumor results

To determine the in *vivo* effect of 3-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosylsulfanyl)-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazole **10** and 1-((4-benzyl-1-oxo-1*H*-phthalazin-2-yl) acetyl)-4-phenyl-thiosemicarbazide **16** on tumor cell growth, Ehrlich Ascites Carcinoma Cells (EAC′s) were treated with or without **10** and **16**.

Cisplatin, which is one of the most effective metallo-anticancer agents, was used as the reference drug in this study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of Ehrlich Ascites Carcinoma Cells (EAC′s). The response parameter was the $IC_{50}$ value, which corresponds to the concentration required for 50 % inhibition of cell viability.

The treatment with **10** and **16** as shown in (Fig.5) produced a significant reduction ($P \leq 0.05$) in tumor mass on days 14 post-inoculation as compared with cisplatin or control. These compounds inhibit the growth

EAC with $IC_{50}$ values of 7.25 and 7.5μM, respectively. These compounds showed slight better cytotoxicity compared to cisplatin ($IC_{50}$= of 9.0 μM).

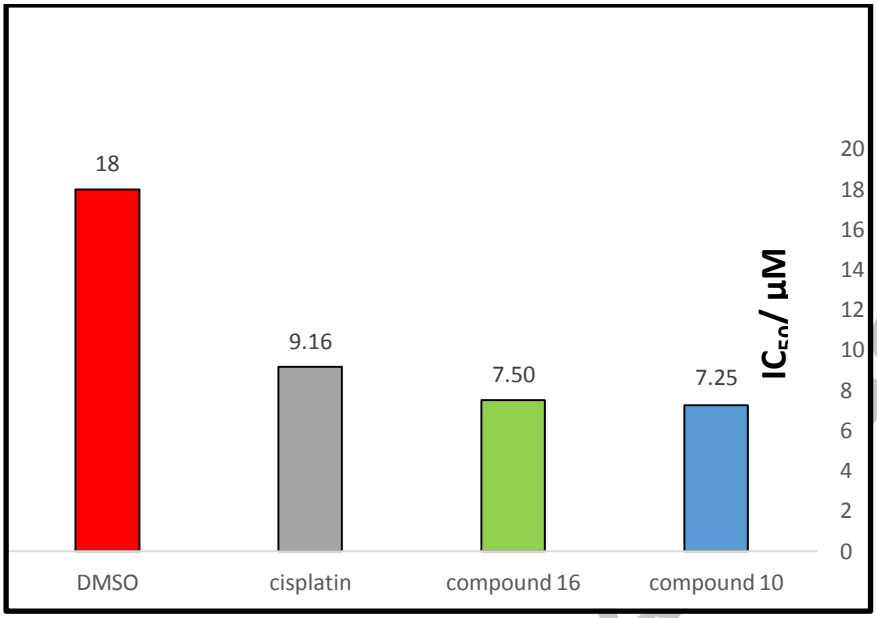



**Fig. 5**. Statistical analysis of the compounds **10** and **16** treatment on solid tumor mass.

## 3. Molecular modeling

We utilized docking simulation studies to substantiate the proposed mode of action of our synthesized compounds and to guide the structural modification to optimize the compounds target interactions.

Study of crystal structures of the EGFR kinase bound to different inhibitors revealed key residues involved in binding all the inhibitors including Leu 718, Val 726, Leu 777, Leu 788, Thr 790, Leu 844, and Asp 855.

The tested compounds were evaluated for their binding affinity to EGFR and their binding energies were calculated using AutodockVina® (Table 1). Comparative docking with the targets crystallized ligands was performed to further validate the docking results and substantiate the compounds mode of action. The results were evaluated based on binding affinity calculation together with cluster size determination and visually

through possible interaction with key residues at the active site. After docking the cluster analysis of the obtained complexes was done. The total number of best-scoring poses analyzed was 30.The procedure successfully reproduced experimentally observed binding modes, and the energy scores for these structurally related binders were in the range from -8.1- -11.1 with the experimentally most active compounds had high but not the highest binding energies. However, with respect to cluster size compounds **16** and **31a** had the largest cluster size which indicates that these compounds are more likely to adopt this binding mode in biological system.

Upon computational docking the inhibitors were found predominantly at the crystallized inhibitor same position (Fig. 6). For the most active compounds, docking poses for compounds **10**, **16** were found to be very close to the co-crystallized inhibitor, however compounds **4**, **31a** occupied the same channel but with a slightly different pose and fewer binding interactions. The reason behind that could be attributed to the dimension of the molecule, being larger and more flexible in compounds **10** and **16** compared to compounds **4** and **31a**.

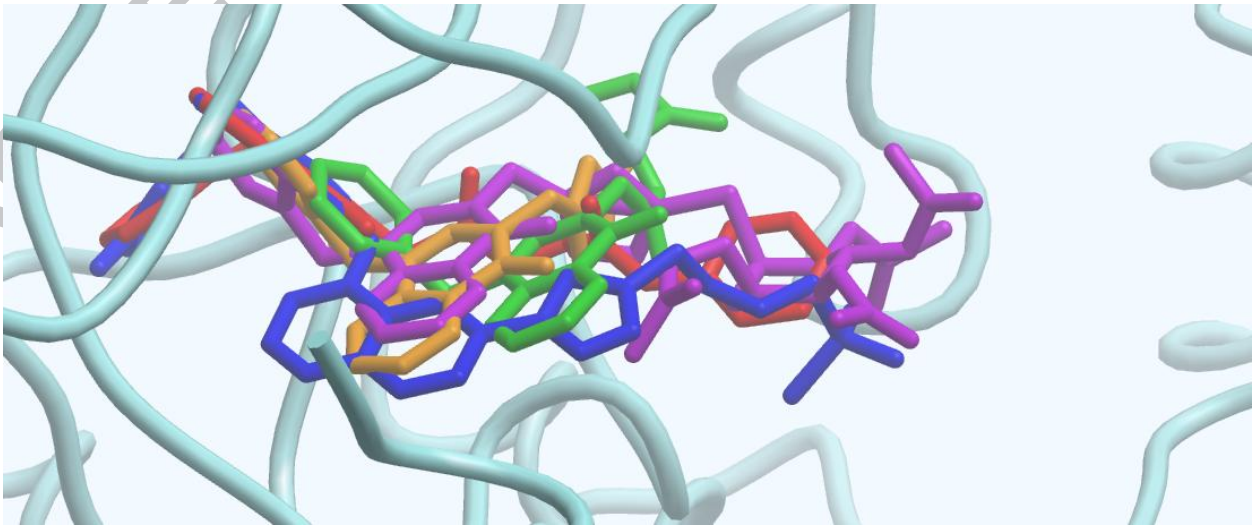


**Fig. 6**. Compounds **16** red, **31a** green, **4** brown, **10** purple, crystallized inhibitor blue, docked in EGFR.

The common amino acids involved in binding all inhibitors include Lys 745, Cys 775, Leu 788, Leu 799, Asp 800, Arg 841 and Thr 854 (Fig. 7).

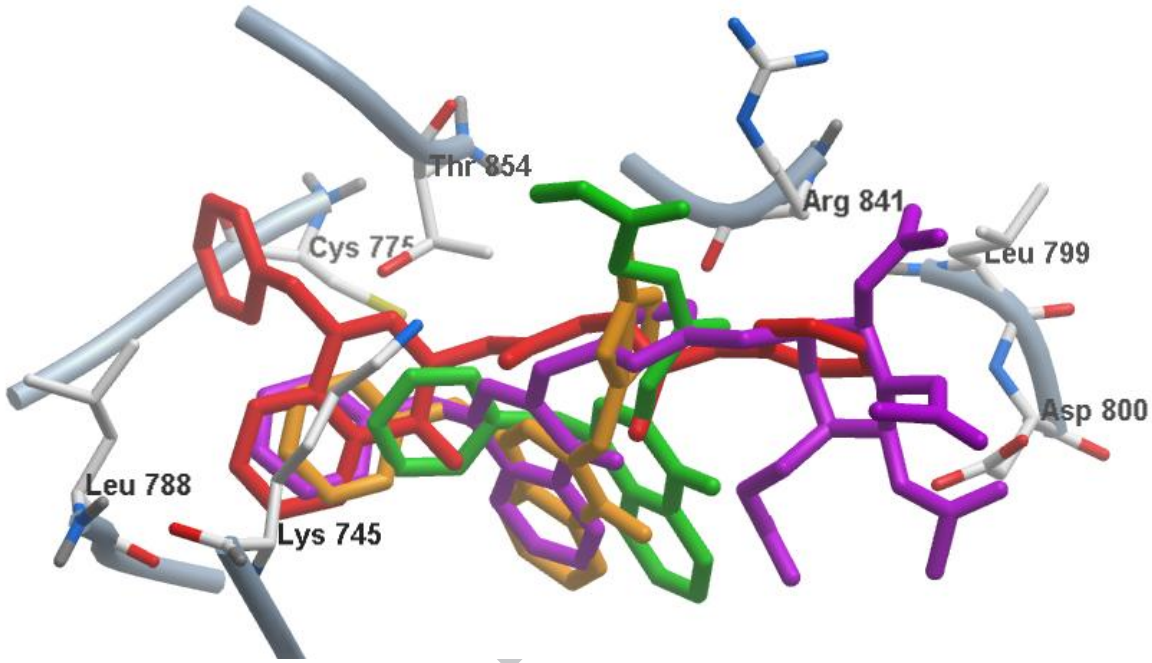



**Fig. 7.** Compounds **16** red, **31a** green, **4** brown, **10** purple, docked in EGFR with key residues involved in ligand binding.

The docking of the two most active compounds, **16** and **31a** was compared to identify any crucial binding requirements for optimum activity. Although, the two compounds showed slightly different binding poses, they are still binding some key residues in common. Compound **16** docked well in the EGFR active site with a docking pattern that allowed possible hydrogen bonding between the two hydrazidenitrogens and Arg 841 and Asn 842 (Fig. 8). One of the phthalazine nitrogens is forming hydrogen bonds with Thr854, and Asp855. Other residues holding the inhibitor tightly in the active site through hydrophobic interactions include Lys 721, Lys 745, Met 766, Leu 777, Leu 788, Thr 790, Leu 799, Asp 800, Leu 844, Thr 854.

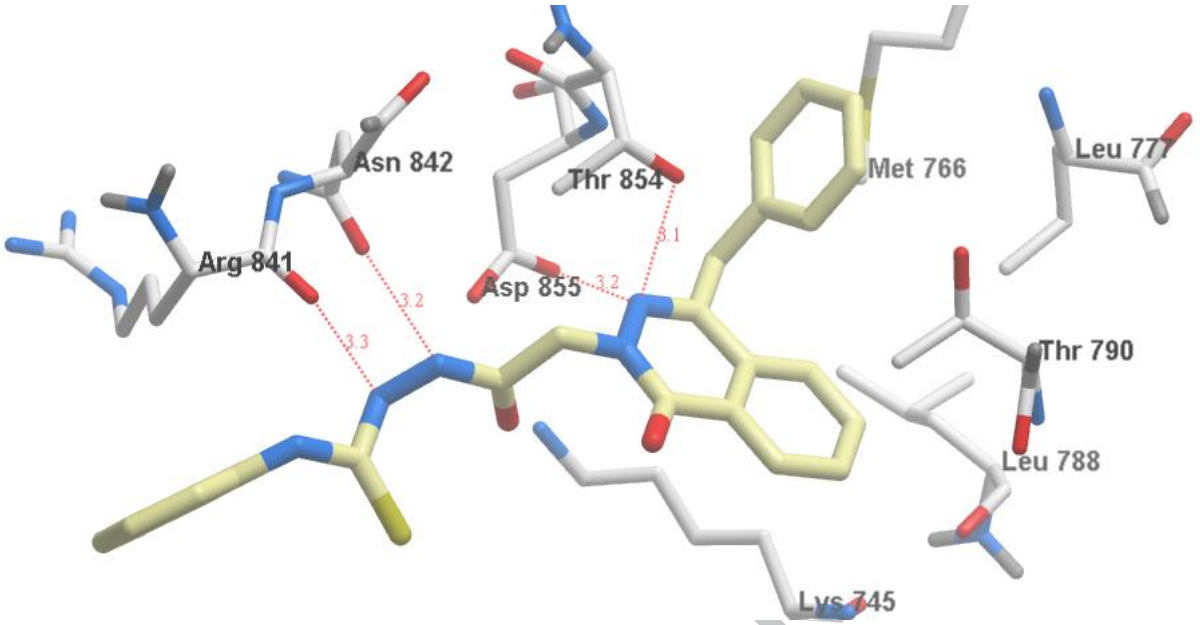



**Fig. 8.** Compound **16** docked in EGFR active site with key residues involved in ligand binding; distances in angstrom is presented by red dotted lines for potential hydrogen bonding.

Figure 9 showing the docking pose of compound **31a** in EGFR active site which similarly to compound **16** was able to bind Arg 841 with its ester and amide oxygen through hydrogen bonding. Compound **31a** established several interactions with residues at the active site namely, Cys 797, Asn842, Leu 844, Thr 854 and Asp 855. The cluster size determination also agreed with the experimental antiproliferative activity.

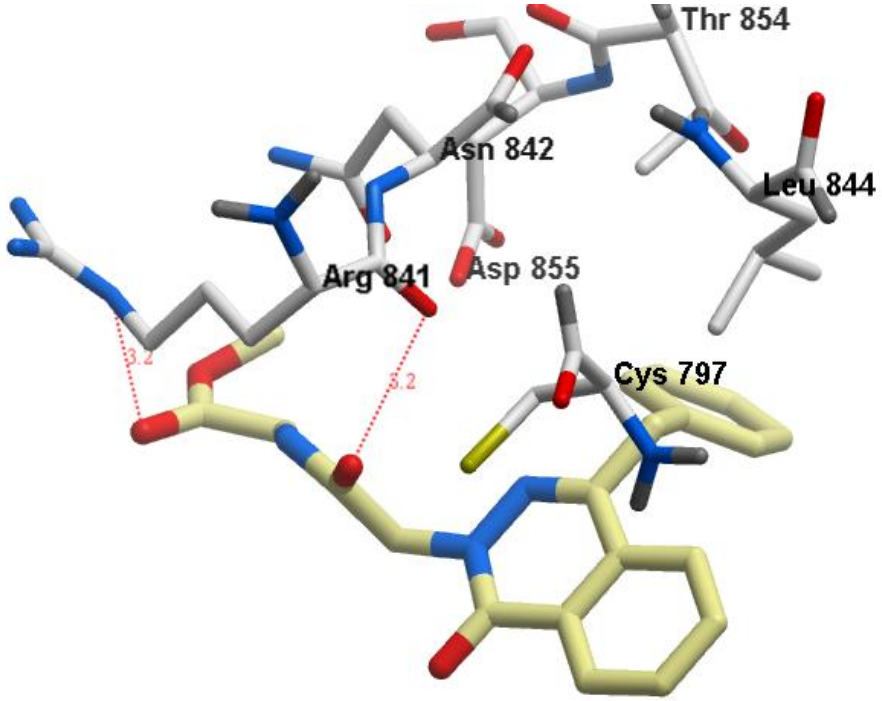



**Fig. 9**. Compound **31a** docked in EGFR active site with key residues involved in ligand binding; distances in angstrom is presented by red dotted lines for potential hydrogen bonding.

Both compounds **16** and **31a** occupied the three main binding regions of EGFR active site (Fig. 10)

Compound **16** ($IC_{50}$7.09µg/mL) adopts a position like that of the well-known inhibitor Gefitinib. The phthalazine ring lies in the pocket formed by Thr790, Gln791, Leu792, and Met793. The benzyl substituent is directed toward the hydrophobic pocket formed by Arg776, Leu777, Arg855, and Phe856. The side chain is positioned at the cleft formed by Ala840, and Arg841.

Compound **31a** ($IC_{50}$5.7µg/mL) shows slightly different pose with its side chain slightly directed toward the cleft where it forms a strong hydrogen bonding with its carboxylate and Arg841. The two most active compounds identified Arg841 as an important residue in the cleft region

for binding of more potent inhibitors. Substitution on the side chain with branched or bulky rings resulted in less active compounds due to steric effect with residues in the cleft region. A chain length of 5-6 atoms containing hydrogen donor or hydrogen acceptor atoms was found optimum for this type of compounds for better binding with the cleft region.

Possible future optimization would focus on the benzyl moiety for a better binding with the hydrophobic pocket and better positioning of the phthalazine ring in its binding pocket.

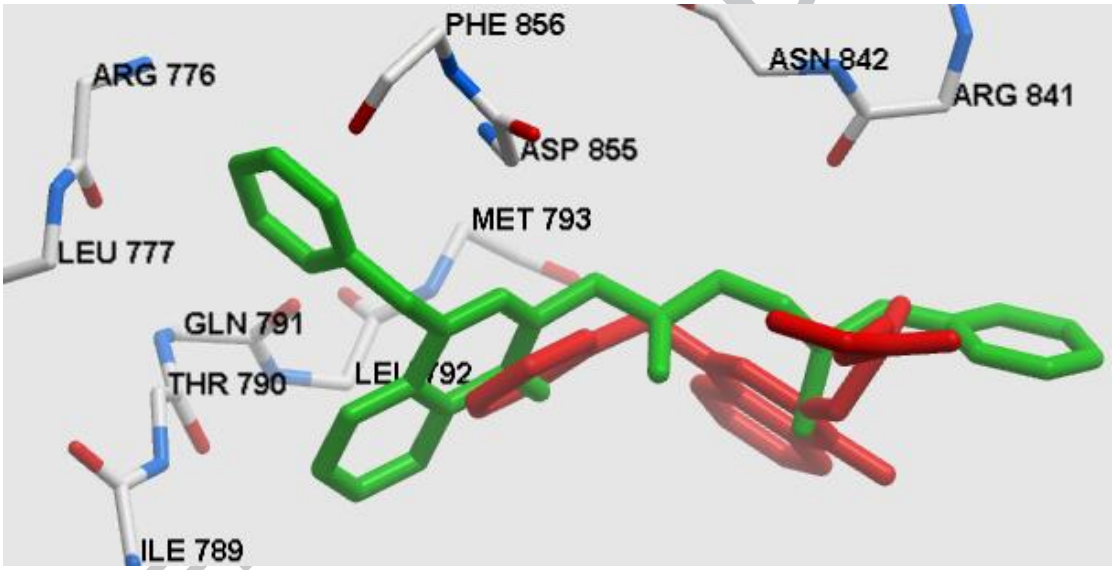



**Fig. 10**. Compound **16** and **31a** docked in EGFR active site and showing interactions with the three major binding pockets.

Molecular docking studies revealed that our synthesized compounds are potential good inhibitors for EGFR, having similar binding mode to the co-crystallized inhibitors. Compounds with a shorter side chain exhibited slightly different conformations while those with longer side chains had similar conformations to the co-crystallized inhibitors. The most active compounds were able to set up stronger interactions with the active site through hydrogen bonding especially with Arg 841.

## 4. Conclusion

In conclusion, a new heterocyclic systems of phthalazines attached to 1,3,4-oxadiazoles, 1,2,4-triazoles, amino acid esters and amines were synthesized and characterized using NMR, IR and elemental analysis. The synthesized compounds showed good activity against hepatocellular carcinoma both *in vitro* and *in vivo*. Molecular docking showed that the synthesized phthalazine derivatives are good inhibitors for EGFR. We were finally able to identify Compounds **4**, **10**, **16**, **31a** as new phthalazine lead compounds added to our previous indole leads for EGFR inhibition. These compounds will be further optimized and tested to get a hit suitable for preclinical development.

## 5. Experimental Part

### General procedures

Melting points were measured with a Buchi 510 melting point apparatus in open capillaries and are uncorrected. Thin layer chromatography (TLC) was done on silica gel 60 $F_{254}$ aluminium. The spots were detected by UV lamp. Microanalysis was performed on Flash EA-1112 insrtument at the microanalytical laboratory, Cairo University, Giza, Egypt. $^{1}$H-NMR and $^{13}$C-NMR spectra were measured on Bruker spectrometer operating at (300 and 400 MHz for $^{1}$H and 75-100 MHz for $^{13}$C). The IR spectra were recorded on Perkin Elmer FTIR spectrometer using KBr.

### 5.1. Chemistry

#### 5.1.1. Synthesis of 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl) acetic acid (2)

To a solution of ester **1** (0.01 mol) in ethanol (95%, 25 mL), sodium hydroxide (0.01 mol /0.5 mL $H_2O$) was added. The reaction mixture was

stirred at room temperature for 3 hrs. Then the mixture was acidified with Conc. HCl, the formed precipitate was filtered off, washed with water, dried and crystalized from methanol to give **2** as white crystals.

Yield: 92.3%. m.p. 196-198$^{o}$C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.32 (s, 2 H, C$H_2$Ph ), 4.87 (s, 2 H, NC$H_2$COOH ), 7.16-8.29 (m, 9 H, Ar*H*), 13.04 (brs, 1 H, O*H* ); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.5 (*C*$H_2$Ph), 52.4 (N*C*$H_2$), 125.9, 126.3, 126.5, 127.3, 128.3, 128.5, 128.8, 131.8, 133.5, 138.0 (12 $C_{aromatic}$), 145.1 (*C*=$N_{Phth.}$), 158.4 (*C*=$O_{Phth.}$), 169.4 (*C*=$O_{acid}$). IR (KBr, ν cm$^{-1}$) 1625 (C=$O_{Phth.}$), 1750 (C=$O_{acid}$), 2500 - 3429 ($OH_{Carboxylic}$), EILR-MS: m/z (%) 65 (20.8), 91 (99.9), 235 (15.5), 250 (99.9), 294 (60 $M^{+\cdot}$). Anal. Calcd for $C_{17}H_{14}N_2O_3$ (294.10): C, 69.38; H, 4.79; N, 9.52; Found C, 69.52; H, 4.74; N, 9.56.

### 5.1.2. Synthesis of 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetohydrazide (3)

A mixture of ethyl 2-(4-benzyl-1-oxophthalazin-2(1*H*)-yl) acetate **(1)** (0.01 mol) and hydrazine hydrate (0.04 mol) in ethanol (30 mL) was refluxed for 4 hrs. On cooling, a solid product was formed, filtered off and recrystallized from ethanol to give **3** as faint yellow crystals.

Yield: 80.51 %, m.p. 184-186 $^{o}$ C; $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.30 (s, 4 H, C$H_2$Ph, -N$H_2$ ), 4.77 (s, 2 H, NC$H_2$-CO), 7.18-8.28 (m, 9 H, Ar*H*), 9.31 (s, 1 H, N*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.6 (*C*$H_2$Ph), 52.1 (N*C*$H_2$CO), 125.7, 126.3, 126.4, 127.6, 128.3, 128.5, 128.9, 131.5, 133.2, 138.0 (12$C_{aromatic}$), 144.9 (*C*=$N_{Phth.}$), 158.5 (*C*=$O_{Phth.}$), 166.4 (*C*=$O_{Hydrazide}$). IR (KBr, ν cm$^{-1}$) 1587 (C=$O_{Phth.}$), 1658 (C=$O_{Hydrazide}$), 3290, 3300 ($NHNH_2$), EILR-MS: m/z (%) 65 (23), 91 (99.9), 249 (99.9), 277 (99.9), 293 (13.4), 308 (9.2 $M^{+\cdot}$). Anal. Calcd for$C_{17}H_{16}N_4O_2$ (308.13): C, 66.22; H, 5.23; N, 18.17; Found C, 66.32; H, 5.28; N, 18.12.

### 5.1.3. Synthesis 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1, 3, 4-oxadiazol-2-thione (4)

To a solution of hydrazide **3** (0.01 mol) in ethanol (25 mL) containing KOH (0.015 mol/0.5 mL $H_2O$), carbon disulphide (0.05 mol) was added and the reaction mixture was refluxed for 3 hrs. The mixture was left to cool to room temperature, and then acidified with Conc. HCl. The formed precipitate was filtered off, washed with water dried and crystalized from absolute ethanol to give **4** as white crystals.

Yield: 95.6 %, m.p. 242-244 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.32 (s, 2 H, C$H_2$Ph), 5.49 (s, 2 H, NC$H_2$), 7.19-8.31 (m, 9 H, Ar*H*), 14.60 (br, 1 H, N$H_{Oxad.}$); $^{13}$C-NMR (DMSO-$d_6$ ,75 MHz) δ 37.5 (*C*$H_2$Ph), 45.2 (N*C*$H_2$), 125.8, 126.0, 126.3, 126.5, 127.1, 128.3, 128.5, 128.7, 128.8, 132.1, 133.8, 137.6 (12 $C_{aromatic}$), 146.3 (*C*=$N_{Phth.}$), 154.8 (*C*-$5_{Oxad.}$), 158.2 (*C*=$O_{Phth.}$), 169.4 (*C*=S). IR (KBr, ν cm$^{-1}$): 1633 (C=$O_{Phth.}$), 3437 (NH). EILR-MS: m/z (%) 65 (24.4), 91 (100), 249 (15.9), 277 (8.2), 350 (29.9 $M^{+\cdot}$). Anal. Calcd for $C_{18}H_{14}N_4O_2S$ (350.08): C, 61.70; H, 4.03; N, 15.99; S, 9.15; Found C, 61.73; H, 3.98; N, 16.02; S, 9.06.

### 5.1.4. General procedure for preparation of compounds 5, 6 and (9-12)

To a solution of oxadiazolethione **4** (0.01 mol) in acetone (20 mL), potassium carbonate (0.015 mol) was added and stirred for 1 hr, then, the appropriate alkyl or glycosyl bromide (**7**,**8**) (0.011 mol) was added. The reaction mixture was stirred at room temperature overnight. The mixture was filtered off and the solvent was removed. The products were purified by crystallization from methanol in case of **5 and 6** or using column chromatography (ethyl acetate/petroleum ether 4:7) in case of **9-12**.

### 5.1.4.1. 2-Allylsulfanyl-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazole (5)

Yield: 99.54 % as white crystals, m.p. 128°C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 3.87 (dd, 2 H, *J*7.8 Hz, $^{2}J$ 0.9 Hz, SC$H_2$), 4.31 ( s, 2 H, C$H_2$Ph), 5.07 (dd, 1 H, $J_{cis}$ *9.0 Hz*, $^{2}J$ 0.9 Hz, *CH=CH*H), 5.26 (dd, 1 H, $J_{trans}\approx$ *16.0Hz*, $^{2}J$ 1.2 Hz, CH=CH*H*), 5.62 (s, 2 H, NC$H_2$), 5.92-5.98 ( m, 1 H, C*H*=$CH_2$), 7.25-8.28 ( m, 9 H, Ar*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 34.5 (S*C*$H_2$), 37.4 (*C*$H_2$Ph), 45.1 (N*C*$H_2$), 119.2, 126.0, 126.4, 126.5, 127.2, 128.3, 128.5, 128.7, 132.1, 132.4, 133.8, 137.7 (14 $C_{\text{aromatic +olefinic}}$),146.2 (*C*=$N_{\text{Phth.}}$),158.2 (*C*=$O_{\text{Phth.}}$),163.6,163.7 (*C*-$2_{\text{Oxad.}}$,*C*-$5_{\text{Oxad.}}$).IR (KBr, ν $cm^{-1}$) 1644 (C=$O_{\text{Phth.}}$). EILR-MS: m/z (%) 65 (17.7),91 (99.9),235 (26.4),249 (99.9),317 (99.9),349 (9.7),390 (99.9 $M^{+}$). Anal.Calcd For $C_{21}H_{18}N_4O_2S$ (390.12): C, 64.60; H, 4.65; N, 14.35; S, 8.21 Found C, 64.85; H, 4.58; N, 14.44; S, 8.19.

### 5.1.4.2. 2-Benzylsulfanyl-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1, 3,4-oxadiazole (6)

Yield: 94.69 % as white crystals, m.p. 94-97 °C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.30 (s, 2 H, C$H_2$Ph), 4.46 (s, 2 H, SC$H_2$), 5.62 (s, 2 H, NC$H_2$), 7.17-8.31 (m, 14 H, Ar*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 35.8 (S*C*$H_2$), 37.4 (*C*$H_2$Ph), 45.1 (N*C*$H_2$), 126.0, 126.5, 127.2, 127.6, 128.3, 128.4, 128.5, 128.7, 128.8, 132.1, 133.8, 136.4, 137.7 (18 $C_{\text{aromatic}}$), 146.2 (*C*=$N_{\text{Phth.}}$), 158.2 (*C*=$O_{\text{Phth.}}$), 163.62, 163.69 (*C*-$5_{\text{Oxad.}}$, *C*-$2_{\text{Oxad.}}$). IR (KBr, ν $cm^{-1}$) 1652 (C=$O_{\text{Phth.}}$).EILR-MS: m/z (%) 65 (29), 91 (99.9), 249 (99.9), 317 (65.7), 349 (14.9), 440 (99.9 $M^{+}$). Anal. Calcd for$C_{25}H_{20}N_4O_2S$ (440.13): C, 68.16; H, 4.58; N, 12.72; S, 7.28 Found C, 68.11; H, 4.63; N, 12.67; S, 7.32.

### 5.1.4.3. 2-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylsulfanyl)-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazole (9)

Yield: 57.29 %, m.p. 70°C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 1.92, 1.96, 1.99 (4s ,12 H, 4 C*H*$_3$CO), 3.96-4.12 (m, 3 H, *H*-5$_{Glc}$, *H*-6$_{Glc}$, *H*-6'$_{Glc}$), 4.31 (s, 2 H, C*H*$_2$Ph), 5.02 (dd, 1 H, $J_{3,4}$ 9.6 ≈ $J_{4,5}$ 9.6 Hz, *H*-4$_{Glc}$), 5.10 (dd, 1 H, $J_{1,2}$ 9.9, $J_{2,3}$ 9.3 Hz, *H*-2$_{Glc}$), 5.40 (dd, 1 H, $J_{3,4}$ 9.6, $J_{2,3}$ 9.3 Hz, *H*-3$_{Glc}$), 5.66-5.69 (m, 3 H, *H*-1$_{Glc}$, NC*H*$_2$), 7.18-8.31 (m, 9 H, Ar*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 20.1, 20.2, 20.3, 20.4 (4 *C*H$_3$), 37.5 (*C*H$_2$Ph), 45.2 (N*C*H$_2$), 61.5 (*C*-6$_{Glc}$), 67.6 (*C*-4$_{Glc}$), 69.7 (*C*-2$_{Glc}$), 72.6 (*C*-3$_{Glc}$), 75.0 (*C*-5$_{Glc}$), 81.9 (*C*-1$_{Glc}$), 126.0, 126.5, 127.2, 128.3, 128.5, 128.7, 132.1, 133.8, 137.7 (12 C$_{aromatic}$), 146.2 (*C*=N$_{Phth.}$), 158.3 (C=O$_{Phth.}$), 160.2 (C-5$_{Oxad.}$), 164.6 (C-2$_{Oxad.}$), 169.0, 169.1, 169.4, 169.8 (4 C=O). IR (KBr, ν cm$^{-1}$) 1660 (C=O$_{Phth.}$), 1752 (C=O$_{Ester}$). EILR-MS: m/z (%) 249 (67), 350 (14), 680 (0.2 M$^+$). Anal.Calcdfor $C_{32}H_{32}N_4O_{11}S$ (680.18): C, 56.46; H, 4.74; N, 8.23; S, 4.71 Found C, 56.52; H, 4.72; N, 8.28; S, 4.76.

### 5.1.4.4. 3-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosylsulfanyl)-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazole (10)

Yield: 57.29%, m.p. 80$^0$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 1.94, 2.00, 2.12 (3s, 12 H, 4 C*H*$_3$CO), 4.00-4.37 (m, 5 H, *H*-5$_{Gal}$, *H*-6$_{Gal}$, *H*-6'$_{Gal}$, C*H*$_2$Ph), 5.21-5.67 (m, 6 H, *H*-1$_{Gal}$, *H*-3$_{Gal}$, *H*-2$_{Gal}$, *H*-4$_{Gal}$, NC*H*$_2$),), 7.18-8.29 (m, 9 H, Ar*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 20.2 (4 *C*H$_3$), 37.5 (*C*H$_2$Ph), 45. 2 (N*C*H$_2$), 61.2 (*C*-6$_{Gal}$), 67.3 (*C*-4$_{Gal}$, *C*-2$_{Gal}$) ,70.6 (*C*-3$_{Gal}$), 74.2 (*C*-5$_{Gal}$), 82.6 (*C*-1$_{Gal}$), 126.0, 126.5, 127.2, 128.3, 128.5, 128.7, 132.0, 133.7, 137.7 (12 *C*$_{aromatic}$), 146.1 (*C*=N$_{Phth.}$), 158.2 (*C*=O$_{Phth.}$), 160.5 (*C*-5$_{oxad}$), 164.6 (*C*-2$_{oxad}$), 169.3, 169.4, 169.7, 169.8 (4*C*=O). IR (KBr, ν cm$^{-1}$) 1661 (C=O$_{Phth.}$), 1751 (C=O$_{Ester}$). EILR-MS: m/z (%)

65(5.7), 91(44), 249 (28), 350 (20), 680 (2.7 $M^+$). Anal. Calcd for $C_{32}H_{32}N_4O_{11}S$ (680.18): C, 56.46; H, 4.74; N, 8.23; S, 4.71 Found C, 56.52; H, 4.72; N, 8.28; S, 4.76.

**5.1.4.5. 3-(2, 3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazol-2-thione (11)**

Yield: 57.29%, m.p. $70^0$C. $^1$H-NMR (DMSO-$d_6$ , 300 MHz) δ 1.91, 1.96, 1.97, 1.98, 2.01 (4s, 12 H, 4 C$H_3$CO), 4.05-4.33 (m , 5 H, *H*-$5_{Glc}$, *H*-$6_{Glc}$, *H*-$6'_{Glc}$) , 5.28 (dd , 1 H, $J_{3,4} \approx J_{4,5}$ 9.9 Hz, *H*-$4_{Glc}$), 5.48 (dd, 1 H,$J_{2,3}$ 9.0, $J_{3,4}$ 9.9 Hz, *H*-$3_{Glc}$) , 5.56-5.59 (m, 3 H, H-$2_{Glc}$, 2 H , NC$H_2$), 6.25 (d,1 H, $J_{1,2}$ 9.3, *H*-$1_{Glc}$), 7.19-8.30 (m , 9 H, Ar*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 20.2, 20.3, 20.4, 20.5 (4 *C*$H_3$), 37.4 (*C*$H_2$Ph), 45.4 (N*C*$H_2$), 61.4 (*C*-$6_{Glc}$), 67.3 (*C*-$4_{Glc}$), 69.4 (*C*-$2_{Glc}$) ,72.1 (*C*-$3_{Glc}$), 73.1 (*C*-$5_{Glc}$), 81.7 (*C*-$1_{Glc}$), 126.1, 126.5, 127.2, 128.3, 128.5, 128.7, 132.1, 133.9, 137.7 (12 $C_{aromatic}$), 146.4 (*C*=$N_{Phth.}$), 158.3 (*C*=$O_{Phth.}$), 158.8 (*C*-$5_{Oxad}$), 168.2, 169.2, 169.5, 170.0 (4 *C*=O), 177.3 (*C*=S). IR (KBr, ν $cm^{-1}$) 1659 (C=$O_{Phth.}$), 1747(C=$O_{Ester}$). EILR-MS: m/z (%) 65 (2.5), 91 (24), 249 (29), 350(9), 680 (1.5 $M^+$). Anal. Calcd for $C_{32}H_{32}N_4O_{11}S$ (680.18): C, 56.46; H, 4.74; N, 8.23; S, 4.71 Found C, 56.52; H, 4.72; N, 8.28; S, 4.76.

**5.1.4.5. 3-(2, 3,4, 6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazol-2-thione (12)**

Yield: 57.29%. m.p. $80^0$C. $^1$H-NMR (DMSO-$d_6$, 300 MHz) δ 2.03, 2.06, 2.11, 2.12 (4s,12 H,4 C$H_3$CO), 3.99-4.10 (m, 3 H , *H*-$5_{Gal}$, *H*-$6_{Gal}$, *H*-$6'_{Gal}$), 4.32 (s, 2 H, C$H_2$Ph), 5.26-5.58 (m, 5 H, *H*-$2_{Gal}$, *H*-$3_{Gal}$, *H*-$4_{Gal}$, NC$H_2$), 6.16 (d, 1 H, $J_{1,2}$ 8.4, H-$1_{Gal}$), 7.16-8.27 (m, 9 H, Ar*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 20.2, 20.3, 20.4, 20.5 (4 *C*$H_3$), 37.5 (*C*$H_2$Ph), 45.3 (N*C*$H_2$), 61.8 (*C*-$6_{Gal}$), 67.6 (*C*-$2_{Gal}$, *C*-$4_{Gal}$), 70.5 (*C*-$3_{Gal}$), 72.7 (*C*-$5_{Gal}$), 82.3 (*C*-$1_{Gal}$), 126.1, 126.5, 127.2, 128.2, 128.3, 128.5, 128.7, 131.5,

132.1, 133.8, 137.7 (12 $C_{aromatic}$), 146.4 ($C$=$N_{Phth.}$), 158.3 ($C$=$O_{Phth.}$), 158.7 ($C$-$5_{oxad}$), 169.3, 169.5, 169.8, 169.9 (4$C$=O), 177.3 ($C$=S). IR (KBr, ν $cm^{-1}$) 1660 (C=$O_{Phth.}$), 1751(C=$O_{Ester}$). Anal. Calcd for $C_{32}H_{32}N_4O_{11}S$ (680.18): C, 56.46; H, 4.74; N, 8.23; S, 4.71 Found C, 56.52; H, 4.72; N, 8.28; S, 4.76.

### 5.1.5. Synthesis 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-4-amino-1,2,4-triazole-3-thione (13)

To a solution of hydrazide **3** (0.01 mol) in ethanol (25 mL) containing KOH (0.015 mol / 0.5 mL $H_2O$), carbon disulphide (0.05 mol) was added and the reaction mixture was refluxed on water bath for 1 hr. The amount of ethanol was increased to (75 mL) and (50 mL) of diethyl ether was added and stirring was continued 6 hrs. The formed precipitate was collected by filtration, dried and used directly for the next step. To the obtained solid (0.01 mol) in $H_2O$ (3 mL), hydrazine hydrate (0.05 mol) was added and the mixture was refluxed for 4 hrs. The contents were cooled, diluted with water and acidified with Conc. HCl. The formed precipitate was collected by filtration, washed with water, dried and crystalized from absolute ethanol to give **13** as white crystals.

Yield: 52.54 %, m.p. 198-200$^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.29 (s, 2 H, C$H_2$Ph), 5.45 (s, 2 H, NC$H_2$), 5.63 (s, 2H, N$H_2$), 7.17-8.28 (m, 9 H, Ar$H$), 13.67 (brs, 1H, N$H$). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.5 ($C$H$_2$Ph), 44.8 (N$C$H$_2$), 125.8, 126.5, 127.3, 128.4, 128.5, 128.7, 131.8, 133.5, 137.8 (12 $C_{aromatic}$), 145.6 ($C$=$N_{Phth.}$), 148.1 ($C$-$5_{Triazol}$), 158.3 ($C$=$O_{Phth.}$), 166.3 ($C$=S). IR (KBr, ν $cm^{-1}$) 1357 (C=S), 1632 (C=$O_{Phth.}$), 3443 (NH). EILR-MS: m/z (%)65 (14.3),91 (97.6),222 (99.9),235 (22.5),249 (99.9), 277 (71.9), 347 (13.6), 364 (99.9 $M^{+\cdot}$). Anal. CalcdFor

$C_{18}H_{16}N_6OS$ (364.11):C, 59.32; H, 4.43; N, 23.06; S, 8.80 Found C, 59.38; H, 4.35; N, 23.13; S, 8.76.

### 5.1.6. Synthesis of 1-((4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetyl)thiosemi-carbazide (14)

To a mixture of hydrazide **3** (0.01 mol) in water (25 mL) containing hydrochloric acid (1.0 mL), ammonium thiocyanate (0.015 mol) was added and the mixture was refluxed for 2 hours until a precipitate is appeared. The mixture was allowed to cool to room temperature and the solid was filtered off, dried, and crystallized from ethanol to give **14** as white crystals.

Yield: 78.68%, m.p. 134-138$^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.32 (s, 2 H, C$H_2$Ph ), 4.91 (s, 2 H, NC$H_2$ ), 7.07-8.27 (m, 11 H, Ar$H$ + N$H_2$) , 9.44 (s, 1 H, N$H$ ), 10.26 (s, 1 H, N$H$ ); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.6 (*C*$H_2$Ph ), 52.7 (N*C*$H_2$), 125.8, 126.3, 126.5, 127.4, 128.3, 128.5, 128.8, 131.7, 132.0, 133.4, 138.0 (12 $C_{aromatic}$), 145.2 (*C*=$N_{Phth.}$), 158.7 (*C*=$O_{Phth.}$) , 166.6 (*C*=O $_{Amide}$ ), 182.0 ( *C*=S ). IR (KBr, ν cm$^{-1}$) 1358 (C=S), 1633 (C=$O_{Phth}$.), 1726 (C=$O_{amide}$), 3281, 3435, 3481 (2NH + $NH_2$). Anal. Calcd for $C_{18}H_{17}N_5O_2S$ (367.11): C, 58.84; H, 4.66; N, 19.06; S, 8.73 Found C, 58.87; H, 4.46; N, 19.14; S, 8.76.

### 5.1.7. Synthesis of 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,2-dihydro-1,2,4-triazole-3-thione (15)

A mixture of hydrazide **3** (1.0 mmol) and thiourea (1.1 mmol) was fused on oil bath at 130 $^{o}$C for 1 hrs. After cooling, the sticky mass was dissolved in (30 mL/8%) sodium hydroxide. The filtrate was acidified with 2.0 N HCl. The formed solid was filtered to give **15** as faint yellow crystals.

Yield: 36.84 %. m.p. 90-92 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.29 (s, 2 H, C$H_2$Ph), 5.34 (s, 2 H, NC$H_2$), 7.18-8.30 (m, 9 H, Ar*H*), 13.38, 13.41 (2s, 2 H, 2N*H*).$^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.6 (*C*$H_2$Ph), 45.4 (N*C*$H_2$), 125.8, 126.4, 126.5, 127.7, 128.4, 128.5, 128.8, 131.8, 133.5, 137.8 (12 $C_{aromatic}$), 145.8 (*C*=$N_{Phth.}$), 148.8 (*C*=$N_{Triazol}$), 158.5 (*C*=$O_{Phth.}$), 166.6 (*C*=S). IR (KBr, ν cm$^{-1}$) 1357 (C=S), 1641 (C=$O_{Phth.}$), 3426 (NH). Anal. Calcd for $C_{18}H_{15}N_5OS$ (349.10): C, 61.87; H, 4.33; N, 20.04; S, 9.18 Found C, 61.83; H, 4.46; N, 20.14; S, 9.12.

### 5.1.8. Synthesis of 1-((4-benzyl-1-oxo-1*H*-phthalazin-2-yl) acetyl)-4-phenyl-thiosemicarbazide (16)

To a solution of the hydrazide **3** (0.01 mol), in absolute ethanol (30 mL), phenyl isothiocyanate (0.012 mol) was added and the reaction mixture was stirred at room temperature for 48 hrs. Precipitate was formed with the slow evaporation of the solvent, filtered off, dried and crystalized from ethanol to give **16** as white crystals.

Yield: 84.48 %). m.p. 164-166 $^{o}$C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.33 (s, 2 H, C$H_2$Ph), 4.98 (s, 2 H, NC$H_2$), 7.16-8.28 (m, 14 H, Ar*H*), 9.60 (brs, 1 H, N*H*), 9.81 (brs, 1 H, N*H*), 10.46 (s, 1 H, N*H*Ph). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.6 (*C*$H_2$Ph), 53.1 (N*C*$H_2$), 125.0, 125.9, 126.2, 126.5, 127.4, 128.1, 128.3, 128.5, 128.9, 131.8, 133.5, 138.0, 139.0 (18 $C_{aromatic}$), 145.4 (*C*=$N_{Phth.}$), 158.9 (*C*=O $_{Phth.}$), 166.9 (*C*=$O_{Amide}$), 181.0 (*C*=S). IR (KBr, ν cm$^{-1}$)1626 (C=$O_{Phth.}$), 1682 (C=$O_{Amide}$), 3454 (NH). EILR-MS: m/z (%) 65 (19.3), 91 (99.9), 235 (20.2), 249 (99.9), 334 (20.6), 425 (29 M-18). Anal. Calcd for $C_{24}H_{21}N_5O_2S$ (443.14): C, 64.99; H, 4.77; N, 15.79; S, 7.23 Found C, 64.92; H, 4.70; N, 15.83; S, 7.18.

### 5.1.9. Synthesis of 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-4-phenyl- 1,2,4-triazol-3-thione (17)

Phenylthiosemicarbazide **16** (0.01 mol) was refluxed in 2 N KOH (20.0 mL) for 4 hours, cooled then, acidified with conc. HCl. The formed precipitate was filtered and crystalized from ethanol to give **17** as white crystals.

Yield: 95.74%. m.p. 138-140 $^{0}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.21 (s, 2 H, C$H_2$Ph), 5.29 (s, 2 H, NC$H_2$), 7.19-8.12 (m, 14 H, Ar*H*), 13.91 (s, 1H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.3 (*C*$H_2$Ph), 45.2 (N*C*$H_2$), 125.7, 126.2, 126.5, 126.9, 127.6, 128.3, 128.4, 128.5, 129.0, 129.2, 131.8, 133.0, 133.5, 136.2, 136.3, 137.7 (18 $C_{aromatic}$), 145.7 (*C*=$N_{Phth.}$), 148.3 (*C*=$5_{Triazol}$), 157.8 (*C*=$O_{Phth.}$), 168.1 (*C*=S). IR (KBr, ν cm$^{-1}$) 1315 (*C*=S), 1648 (C=$O_{Phth.}$), 3430 (NH). EILR-MS: m/z (%)65 (9.8), 91 (33.5), 235 (8.4), 249 (4.3), 334 (10.9), 425 (99.9 $M^+$). Anal. Calcd for $C_{24}H_{19}N_5OS$ (425.13): C, 67.74; H, 4.50; N, 16.46; S, 7.54 Found C, 67.79; H, 4.56; N, 16.51; S, 7.49.

### 5.1.10. Synthesis of 5-(4-benzyl-1-oxo-1H-phthalazin-2-ylmethyl)-3-benzylsulfanyl-4-phenyl-1,2,4-triazole (18)

A mixture of 1,2,4-triazole **17** (0.01 mol) and potassium carbonate (0.015 mol) in acetone (20 mL) was stirred for 1 hr, then, benzyl chloride (0.011 mol) was added. The reaction mixture was stirred at room temperature overnight. The mixture was filtered off and the solvent was removed. The product was purified by crystallization from butanol to give **18** as faint yellow crystals.

Yield: 74.16 %. m.p. 158-160$^{o}$C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.19 (s, 2 H, SC$H_2$), 4.34 (s, 2 H, C$H_2$Ph), 5.39 (s, 2 H, NC$H_2$), 7.11-8.14 (m, 19 H, Ar*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 36.2 (S*C*$H_2$), 37.3 (*C*$H_2$Ph), 44.7 (N*C*$H_2$), 120.6, 120.7, 125.6, 126.3, 126.5, 126.8, 127.1, 127.4, 128.4, 128.5, 128.9, 129.4, 129.7, 131.7, 132.4, 133.4, 137.0,

137.8 (24 $C_{aromatic}$), 145.5 ($C$=$N_{Phth.}$), 150.3, 151.8 ( 2$C_{Triazol}$), 157.8 ($C$=$O_{Phth.}$). IR (KBr,ν $cm^{-1}$) 1647 (C=$O_{Phth.}$). Anal.Calcd for $C_{31}H_{25}N_5OS$ (515.18): C, 72.21; H, 4.89; N, 13.58; S, 6.22 Found C, 72.17; H, 4.82; N, 13.63; S, 6.27.

### 5.1.11. Synthesis of 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N*'-(β-D-glucopyranosyl)acetohydrazide (19)

A mixture of hydrazide **3** (0.01 mol), α-D-glucose (0.01 mol), and few drops of glacial acetic acid in ethanol (30 mL) was heated under reflux for 8 hrs. The reaction mixture was allowed to cool and the solid was filtered off, dried, and crystallized from ethanol to give **19** as white crystals.

Yield: 78.68 %, m.p. 172-174$^{o}$C; $^{1}$H-NMR (DMSO-$d_6$+$D_2O$, 300 MHz) δ 3.01-3.77 (m, 6 H, $H$-$2_{Glu}$,$H$-$3_{Glu}$, $H$-$4_{Glu}$, $H$-$5_{Glu}$, $H$-$6_{Glu}$,$H$-$6'_{Glu}$ **)**, 3.85 (d, 1 H, $J_{2,3}$ 9Hz, $H$-$1_{Glu}$), 4.28 (s, 2 H, C$H_2$Ph), 5.04-5.29 (2 d, 1 H, $J$17.1 Hz, NC$H_2$), 7.18-8.28 (m, 9 H, Ar$H$); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 38.4 ($C$H$_2$Ph), 55.2 (NC$H_2$), 64.3, 64.9, 73.2, 73.8, 79.5, 94.0 (6 $C_{Glu}$), 128.6, 128.7, 129.2, 129.4, 130.4, 131.2, 131.3, 131.4, 131.7, 134.5, 134.6, 136.2 (12 $C_{aromatic}$), 147.6 ($C$=$N_{Phth.}$), 169.7, 173.6 (2$C$=O). IR (KBr, ν $cm^{-1}$) 1577 (C=$N_{Phth.}$), 1627 (C=$O_{Phth.}$), 1693 (C=$O_{Amide}$), 3747 - 3441 (OH, NH). Anal. Calcd for $C_{23}H_{26}N_4O_7$ (470.18): C, 58.72; H, 5.57; N, 11.91 Found C, 58.64; H, 5.64; N, 11.87.

### 5.1.12. Synthesis of 2-(4-benzyl-1-oxo-1H-phthalazin-2-yl)-*N*-(1,3-dioxoisoin-dolin-2-yl)acetamide (20)

A mixture of hydrazide **3** (1.0 mmol) and phthalic anhydride (1.0 mmol) in 15 mL acetic acid was refluxed for 8 hrs then cooled to room temperature. The resultant solid was filtered off, dried and crystalized from butanol to give **20** as white crystals.

Yield: 53.62 %. m.p. 232-234 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.32 (s, 2 H, C$H_2$Ph), 5.11 (s, 2 H, NC$H_2$), 7.17-8.30 (m, 13 H, Ar*H*), 11.08 (s, 1 H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.6 (*C*H$_2$Ph), 51.4 (N*C*H$_2$), 123.7, 125.8, 126.4, 127.4, 128.3, 128.5, 128.8, 129.4, 131.7, 133.4, 135.2, 137.9 (18$C_{aromatic}$), 145.4 (*C*=N$_{Phth.}$), 158.5 (*C*=O$_{Phth.}$), 164.8, 166.8 (3*C*=O). IR (KBr, ν cm$^{-1}$) 1666, 1749 (4 C=O**)**, 3440 (NH).Anal. Calcd for $C_{24}H_{18}N_4O_4$ (438.13): C, 68.49; H, 4.14; N, 12.78 Found C, 68.53; H, 4.22; N, 12.68.

### 5.1.13. General procedure for preparation of compounds 21 and 22

A mixture of hydrazide **3** (1.0 mmol) and benzoyl chloride (1.1 mmol) was refluxed in pyridine (20 mL) for 1 hr, then poured on ice and acidified with conc. HCl to give **21**. Or refluxed in ethanol for 3 hrs to give **22** on cooling. Both products were crystalized from ethanol to give **21** as faint yellow crystals and **22** as white crystals.

#### 5.1.13.1. *N*'-(2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl) acetyl)benzohydrazide (21)

Yield: 73.13 %. m.p. 222-224 $^{o}$C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.30 (s, 2 H, C$H_2$Ph), 4.95 (s, 2 H, NC$H_2$), 7.18-8.30 (m, 14 H, Ar*H*), 10.31 (s, 1H, N*H*), 10.48 (s, H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.6 (*C*H$_2$Ph), 51.8 (N*C*H$_2$), 125.8, 126.3, 126.5, 127.4, 127.5, 128.3, 128.4, 128.5, 128.8, 131.7, 131.8, 132.3, 133.4, 138.0 (18 $C_{aromatic}$), 145.1 (*C*=N$_{Phth.}$), 158.5 (*C*=O$_{Phth.}$), 165.3, 166.4 (2 *C*=O). IR (KBr, ν cm$^{-1}$) 1605 (C=O$_{Phth}$), 1650 (2 C=O), 3453 (N*H*). Anal. Calcd for $C_{24}H_{20}N_4O_3$ (412.15): C, 69.89; H, 4.89; N, 13.58 Found C, 69.96; H, 4.75; N, 13.47.

#### 5.1.13.2. 2-Phenyl-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-1,3,4-oxadiazol (22)

Yield: 23.8 %. m.p. 96-98 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.32 (s, 2 H, C$H_2$Ph), 4.96 (s, 2 H, NC$H_2$), 7.18-8.29 (m, 14 H, Ar*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.5 (*C*$H_2$Ph), 52.4 (N*C*$H_2$), 125.9, 126.3, 126.5, 127.2, 128.2, 128.5, 128.8, 131.9, 133.6, 137.9 (18 $C_{aromatic}$), 145.4, 158.4, 168.0 (3*C*=N+*C*=$O_{Phth.}$). IR (KBr, ν cm$^{-1}$) 1734 (C=$N_{Oxad.}$), 1651 (C=O $_{Phth.}$). Anal. Calcd for $C_{24}H_{18}N_4O_2$. (394.14): C, 73.08; H, 4.60; N, 14.20 Found C, 73.14; H, 4.75; N, 14.28.

### 5.1.14. Synthesis of 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N´*-phenylacetohydrazide (23)

A mixture of **20** (1.0 mmol) and phenyl hydrazine (1.0 mmol) in 15 mL ethanol containing few drops of acetic acid was refluxed for 4 hrs then cooled to room temperature. The resultant solid was filtered off, dried and crystalized from dimethyl formamide to give **23** as white crystals.

Yield: 55.67 %. m.p. 258-260 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 2.87 (d, 1 H, N*H*Ph), 4.32 (s, 2 H, C$H_2$Ph), 5.01 (s , 2 H, NC$H_2$), 7.14-8.30 (m,14 H, Ar*H*), 10.37 (s, 1 H, CON*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.7 (*C*$H_2$Ph), 51.8 (N*C*$H_2$), 125.8, 126.4, 126.5, 127.6, 128.3, 128.4, 128.6, 128.9, 129.1, 131.7, 133.4, 135.0, 138.0 (18$C_{aromatic}$), 145.1(*C*=$N_{Phth.}$), 158.6 (*C*=$O_{Phth.}$), 165.9 (*C*=$O_{Amide}$). IR (KBr, ν cm$^{-1}$) 1621 (C=$O_{Phth.}$), 1657 (C=$O_{Amide}$), 3195 (NH). Anal. Calcd for $C_{23}H_{20}N_4O_2$ (384.16): C, 71.86; H, 5.24; N, 14.57 Found C, 71.93; H, 5.19; N, 14.62.

### 5.1.15. Synthesis of ethyl 3-(2-(2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetyl)hydrazono)butanoate (24)

To a mixture ofhydrazide **3** (0.01 mol) in ethanol (15 ml), ethylacetoacetate (0.01 mol) was added and the mixture was refluxed for 6 hrs. The mixture was allowed to cool to room temperature and the

separated solid was filtered off, dried, and crystallized from ethanol to give **24** as white crystals.

Yield: 29.36 %. m.p. 120-122 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 1.21-1.24 (m, 3 H, $CH_2C\underline{H}_3$), 1.97 (s, 3 H, C$H_3$), 3.37 (s, 2 H, C$H_2$COEt), 4.14 (q, 2 H, OC$\underline{H}_2$CH3), 4.32 (s, 2 H, C$H_2$Ph), 5.15 (s, 2 H, *NC*$H_2$), 7.20-8.29 (m, 9 H, Ar*H*), 10.72 (s, 1 H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 14.5 ($CH_3$), 16.8 ($CH_3$), 38.1 (*C*$H_2$Ph), 44.5 (*C*$H_2$), 52.8 (NC$H_2$), 60.9 (OC$H_2$), 126.3, 126.9, 127.0, 128.0, 128.8, 129.0, 129.4, 132.2, 133.9, 138.6 (12$C_{aromatic}$), 145.4 (*C*=N), 148.5 (*C*=$N_{Phth.}$), 159.1 (*C*=$O_{Phth.}$), 169.1 (*C*ONH), 169.9 (C=$O_{Ester}$). IR (KBr, ν cm$^{-1}$) 1655 (*C*=$O_{Phth.}$), 1684 (C=O), 1731 (C=$O_{Ester}$), 3444 (NH). Anal. Calcd for $C_{23}H_{24}N_4O_4$ (420.18): C, 65.70; H, 5.75; N, 13.33 Found C, 65.86; H, 5.68; N, 13.27.

### 5.1.16. Synthesis of ethyl 2-(4-amino-5-((4-benzyl-1-oxo-1*H*-phthalazin-2-yl)methyl)-4*H*-1,2,4-triazol-3-ylsulfanyl)acetate (25)

To a solution of 1,2,4-triazole **13** (0.01 mol) in ethanol (20 mL), potassium carbonate (0.015 mol) was added, then ethyl chloroacetate (0.011 mol) was added. The reaction mixture was refluxed for 6 hours. The mixture was filtered off and the solvent was removed. The product was purified by crystallization from ethanol to give **25** as white crystals.

Yield: 16.13 %. m.p. 128-130 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 1.17 (t, 3 H, *J* 8.0 Hz, C$H_3$), 4.04 (s, 2 H, SC$H_2$), 4.09 (q, 2 H, $\underline{CH_2}CH_3$), 4.32 (s, 2 H, C$H_2$Ph), 5.50 (s, 2 H, NC$H_2$), 6.05 (s, 2 H,N$H_2$), 7.21-8.30 (m, 9 H, Ar*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 14.4 ( $CH_3$), 33.4 (S*C*$H_2$), 38.1 (*C*$H_2$Ph), 44.9 (N*C*$H_2$), 61.6 (O*C*$H_2$), 126.3, 127.0, 128.0, 128.9, 129.0, 129.3, 132.3, 134.0, 138.3 (12$C_{aromatic}$), 146.2 (C=$N_{Phth.}$), 151.7, 152.7 (2 *C*=$N_{Triazol}$), 158.9 (*C*=$O_{Phth.}$), 168.9 (*C*=$O_{Ester}$). Anal. Calcd for

$C_{22}H_{22}N_6O_3S$ (450.15): C, 58.65; H, 4.92; N, 18.65; S, 7.12 Found C, 58.56; H, 4.86; N, 18.54; S, 7.19.

### 5.1.17. Synthesis of 3-Benzylsulfanyl-5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)- 4-amino - 1,2,4-triazole (26)

To a solution of 1,2,4-triazole **13** (0.01 mol) in ethanol (20 mL), potassium carbonate (0.015 mol) was added and stirred for 1 hour, then benzyl chloride (0.011 mol) was added. The reaction mixture was stirred at room temperature overnight. The mixture was filtered off and the solvent was removed. The product was purified by crystallization from methanol to give **26** as faint yellow crystals.

Yield: 73.33 %. m.p. 154-156 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.30 (s, 2 H, C$H_2$Ph), 4.39 (s, 2 H, SC$H_2$Ph), 5.51 (s, 2 H, NC$H_2$), 6.01 (s, 2 H, N$H_2$), 7.18-8.30 (m, 14 H, Ar*H*); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 34.7 (SC$H_2$Ph), 37.6 (*C*$H_2$Ph), 44.5 (NC$H_2$), 125.8, 126.4, 126.5, 127.2, 127.4, 128.3, 128.4, 128.5, 128.7, 128.9, 131.8, 133.5, 137.5, 137.8 (18 $C_{aromatic}$), 145.6 (*C*=$N_{Phth.}$), 151.6, 152.2 (2 C=$N_{Triazol.}$), 158.3 (C=$O_{Phth.}$). IR (KBr, ν $cm^{-1}$) 1657 (C=$O_{Phth.}$), 3307, 3436 ($NH_2$). Anal.Calcd for $C_{25}H_{22}N_6OS$ (454.16): C, 66.06; H, 4.88; N, 18.49; S, 7.05 Found C, 66.15; H, 4.96; N, 18.36; S, 7.13.

### 5.1.18. Synthesis of 4-Benzyl-2-((6-hydroxy- [1,2,4] triazolo[3,4-b]-1,3,4-thiadiazol-3-yl)methyl)phthalazin-1(2H)-one (27)

A mixture of 1,2,4-triazole **13** (1.0 mmol) and urea (1.1 mmol) was fused on oil bath at 130 $^{o}$C for 1 hr. After cooling, the sticky mass was dissolved in (30 mL, 8%) sodium hydroxide. The filtrate was acidified with 2.0 N HCl. The formed solid was filtered off and crystalized from ethanol to give **29** as yellow crystals.

Yield: 65.33 %, m.p. 158-160 $^{o}$C. $^{1}$H-NMR (DMSO + $D_2O$-$d_6$, 300 MHz) δ 4.33 (s, 2 H, C$H_2$Ph), 5.44 (s, 2 H, NC$H_2$), 7.17-8.29 (m, 9 H, Ar*H*), 13.62 (brs, 1H, O*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.5 (*C*$H_2$Ph), 44.8 (N*C*$H_2$), 125.8, 126.5, 127.3, 128.3, 128.4, 128.5, 128.7, 131.8, 133.6, 137.8, 137.9, 145.6 (16 $C_{aromatic}$), 158.3 (C=$O_{Phth.}$). IR (KBr, ν cm$^{-1}$) 1634 (C=$O_{Phth.}$), 3435 (OH). EILR-MS: m/z (%) 91 (99.9), 178 (76.3), 222 (99.9), 249 (99.9), 364 (95.6), 390 (1.3 $M^{+)}$.Anal. Calcd for $C_{19}H_{14}N_6O_2S$ (390.09): C, 58.45; H, 3.61; N, 21.53; S, 8.21 Found C, 58.56; H, 3.72; N, 21.62; S, 8.17.

### 5.1.19. General procedure for preparation of compounds 28, 29

A mixture of 1,2,4-triazole **13** (0.01 mol), aromatic aldehyde namely benzaldehyde and salicaldehyde (0.01 mol) in ethanol (20 mL) containing few drops of acetic acid was refluxed for 10minutes to give white precipitates. The precipitates were filtered off and recrystallized from butanol to give **28** and **29**.

#### 5.1.19.1. 5-(4-Benzyl-1-oxo-1H-phthalazin-2-ylmethyl)-4-(benzylideneamino)-1,2,4-triazole-3-thione (28)

Yield: 81.95 % as white crystals, m.p. 216 $^{o}$C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.22 (s, 2 H, C$H_2$Ph), 5.59 (s, 2 H,1 NC$H_2$), 7.16-8.29 (m, 14 H, Ar*H*), 9.94 (s, 1H, N=C*H*), 14.05 (s, 1H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.4 (*C*$H_2$Ph), 44.5 (N*C*$H_2$), 125.9, 126.5, 127.3, 128.3, 128.4, 128.5, 128.9, 131.8, 132.0, 132.5, 133.7, 137.8(18 $C_{aromatic}$), 145.9, 147.2, 158.4, 161.9 (3*C*=N + *C*=$O_{Phth.}$), 162.6 (*C*=S). IR (KBr, ν cm$^{-1}$) 1355 (C=S), 1644 (C=$O_{Phth.}$), 3105 (NH). Anal. Calcd for $C_{25}H_{20}N_6OS$ (452.14): C, 66.35; H, 4.45; N, 18.57; S, 7.09 Found C, 66.50; H, 4.53; N, 18.54; S, 7.19.

### 5.1.19.2. 5-(4-benzyl-1-oxo-1*H*-phthalazin-2-ylmethyl)-4-(2-hydroxybenzylide-neamino)-1,2,4-triazole-3-thione (29)

Yield: 57.89 % as white crystals, m.p. 212-214 $^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.23 (s, 2 H, C$H_2$Ph), 5.57 (s, 2 H, NC$H_2$), 7.21-8.29 (m, 13 H, Ar*H*), 9.00 (s, 1H, N|=C*H*), 10.29 (s, 1H, O*H*), 13.94 (s, 1H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 37.4 (*C*$H_2$Ph), 44.5 (N*C*$H_2$), 125.8, 126.4, 126.5, 126.8, 127.2, 128.2, 128.4, 128.5, 130.8, 131.8, 133.2, 133.6, 134.0, 137.7 (18 $C_{aromatic}$), 145.7, 147.1, 158.6 (3*C*=N+*C*=$O_{Phth.}$), 162.7 (*C*=S). IR (KBr, ν cm$^{-1}$) 1364 (C=S), 1646 (C=$O_{Phth.}$), 3105 (NH), 3432 (OH). Anal. Calcd for $C_{25}H_{20}N_6O_2S$. (468.14): C, 64.09; H, 4.30; N, 17.94; S, 6.84 Found C, 64.16; H, 4.45; N, 17.88; S, 6.78.

### 5.1.20. General procedure for Azide-coupling of amino acids methyl esters and amines

To a cold solution (-5 $^{o}$C) of hydrazide **3** (1.6 mmol) in acetic acid (12 ml), hydrochloric acid (5 N, 6 mL), and water (50 mL), cold solution (0$^{o}$C) of sodium nitrite (0.14 g, 2.0 mmol) in water (6 mL) was added portion wise. The mixture was stirred at the same temperature for 30 minutes then the azide was extracted with cold ethyl acetate, and washed with cold 5 % $NaHCO_3$ and water. After drying over anhydrous sodium sulphate, the azide **30** was used without further purification in the next step. The appropriate amino acid methyl ester hydrochloride or aromatic amine (1.8 mmol) was stirred in ethyl acetate (50 mL) containing triethyl amine (0.2 mL) at 0$^{o}$C for 20 minutes. The formed triethyl amine hydrochloride was filtered off and the filtrate was added to the previously prepared cold solution of azide **30**.After that, the mixture was kept 12 hrs in the refrigerator and then at room temperature for another 12 hours then was washed with 5% $NaHCO_3$ and water and the organic layer was dried over anhydrous sodium sulphate. The solvent was evaporated under

vacuum and the residue was crystallized from ethyl acetate-petroleum ether to give the respective product.

### 5.1.20.1. Methyl 2-(2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)acetamido)acetate (31a)

Yield: 24.57 % as white crystals, m.p. 154$^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 3.66 (s, 3H, OC$H_3$),), 3.93 (s, 2 H, C$H_{2Gly}$), 4.31 (s, 2 H, C$H_2$Ph), 4.86 (s, 2 H, NC$H_2$), 7.20-8.28 (m, 9 H, Ar*H*), 8.55 (s, 1 H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 38.1 (*C*H$_2$Ph), 41.1 (*C*H$_{2Gly}$), 52.2 (O*C*H$_3$), 53.5 (N*C*H$_2$), 126.3, 126.9, 127.0, 128.1, 128.9, 129.0, 129.4, 132.1, 133.8, 138.5 (12 $C_{aromatic}$), 145.5 (*C*=N $_{Phth.}$), 159.0 (*C*=O$_{Phth.}$), 168.0 (*C*=O$_{Amide}$), 170.6 (*C*=O$_{Ester}$). IR (KBr, ν cm$^{-1}$) 1648 (C=O$_{Phth.}$)**,** 1674 (*C*=O$_{Amide}$), 1761 (C=O$_{Ester}$), 3444 (NH). Anal. Calcd for $C_{20}H_{19}N_3O_4$ (365.14): C, 65.74; H, 5.24; N, 11.50 Found C, 65.70; H, 5.19; N, 11.55.

### 5.1.20.2. Methyl 2-(2-(4-benzyl-1-oxo-1H-phthalazin-2-yl)acetamido)-4-methylpentanoate (31b)

Yield: 63.97 % as white crystals, m.p. 120-122$^{o}$C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 0.90 (d, 6H,*J* 8 Hz, 2C$H_{3Leu}$), 1.58-1.70 (m, 3H, C$H_2$+C$H_{Leu}$),3.65 (s, 3H, OC$H_3$), 4.30 (s, 2 H, C$H_2$Ph), 4.41-4.42 (m, 1H, NC$H_{Leu}$), 4.79-4.92 (2 d, 2 H,$J_{Gem}$ 16 Hz,NC$H_2$), 7.19-8.29 (m, 9 H, Ar*H*), 8.58 (brs,1H,N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 21.9 (2 *C*H$_3$), 23.1 (*C*H$_{Leu}$), 24.7 (*C*H$_{2Leu}$), 38.2 (*C*H$_2$Ph), 50.9 (N*C*H$_{Leu}$), 52.3 (O*C*H$_3$), 53.3 (N*C*H$_2$), 126.2, 126.9, 128.1, 128.8, 128.9, 129.4, 132.1, 133.8, 138.5 (12 $C_{aromatic}$), 145.4 (*C*=N$_{Phth.}$), 159.0 (*C*=O$_{Phth.}$), 167.5 (*C*=O$_{Amide}$), 173.2(*C*=O$_{Ester}$). IR (KBr, ν cm$^{-1}$) 1648 (C=O$_{Phth.}$), 1741(C=O$_{Ester}$), 3424 (NH).Anal. Calcd for $C_{24}H_{27}N_3O_4$ (421.20): C, 68.39; H, 6.46; N, 9.97 Found C, 68.47; H, 6.37; N, 9.91.

### 5.1.20.3. Methyl 2-(2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl) acetamido)-3-hydroxypropanoate (31c)

Yield: 21 % as white crystals, m.p. 101-102°C. $^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 3.67-3.76 (m, 5 H, OC$H_3$, $CH_{2Ser}$), 4.30 (s, 2 H, C$H_2$Ph), 4.46 (m, 1H, $CH_{Ser}$), 4.73 (brs, 1 H, O*H*), 4.91(s, 2 H, NC$H_2$), 7.19-8.27 (m, 9 H, Ar*H*), 8.57 (brs, 1 H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 38.1 (*C*$H_2$Ph), 52.3 (O*C*$H_3$), 53.4 (N*C*$H_2$),55.3 (N*C*$H_{Ser}$), 61.9 (*C*$H_2$O$H_{Ser}$), 126.2, 126.9, 127.0, 128.0, 128.8, 129.0, 129.3, 132.1, 133.8, 138.5 (12 $C_{aromatic}$),145.4 (C=N $_{Phth.}$), 159.0 (C=$O_{Phth.}$), 167.6 (*C*=$O_{Amide}$),171.4 (C=O $_{Ester}$).IR (KBr, ν $cm^{-1}$) 1645 (C=$O_{Phth.}$),1662 (C=$O_{Amide}$), 1736 (C=$O_{Ester}$), 3425 (NH). Anal. Calcd for $C_{21}H_{21}N_3O_5$ (395.15): C, 63.79; H, 5.35; N, 10.63 Found C, 63.70; H, 5. 29; N, 10.56.

### 5.1.20.4. 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N*-benzyl-acetamide (32a)

Yield: 47.58 % as white crystals, m.p. 148-150°C.$^{1}$H-NMR (DMSO-$d_6$, 300 MHz) δ 4.33 (s, 2 H, C$H_2$Ph), 4.36 (s, 2H, NHC$H_2$), 4.87 (s, 2 H, NC$H_2$), 7.20-8.30 (m, 14 H, Ar*H*), 8.64 (brs, 1H, N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 38.2 (*C*$H_2$Ph), 42.7 (NH*C*$H_2$), 54.0 (N*C*$H_2$), 126.2, 126.9, 127.0, 127.2, 127.5, 127.7, 128.2, 128.7, 128.9, 129.0, 129.4, 132.1, 133.7, 138.5, 139.7 (18 $C_{aromatic}$), 145.5 (*C*=$N_{Phth.}$), 159.1 (*C*=$O_{Phth.}$), 167.5 (*C*=$O_{Amide}$). IR (KBr, ν $cm^{-1}$) 1644 (C=$O_{Phth.}$), 1666 (*C*=$O_{Amide}$), 3376 (NH).EILR-MS: m/z (%) 91(99.9), 222 (99.9), 237 (99.9), 249 (99.9), 277 (99.9), 383 (99.9 $M^+$) Anal. Calcd for $C_{24}H_{21}N_3O_2$ (383.16): C, 75.18; H, 5.52; N, 10.96 Found C, 75.26; H, 5.45; N, 10.85.

### 5.1.20.5. 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N*-cyclohexylacetamide (32b)

Yield: 61.16 % as white crystals, m.p. 180-182°C. $^1$H-NMR (DMSO-$d_6$, 300 MHz) δ 1.10-1.83 (m, 10 H, 5 C$H_{2Cyclohexcyl}$), 3.59 (brs, 1H, C*H*), 4.30 (s, 2 H, C$H_2$Ph), 4.68 (s, 2 H, NC$H_2$), 7.20-8.27 (m, 10 H, Ar*H*+N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 25.0 (2*C*H$_2$), 25.7 (*C*H$_2$),32.9 (2*C*H$_2$), 38.1 (*C*H$_2$Ph),48.3 (NH*C*H), 53.7 (*NC*H$_2$), 126.2, 126.9, 128.2, 128.9, 129.0, 129.4, 132.0, 133.7, 138.6 (12 $C_{aromatic}$), 145.2 (*C*=N$_{Phth.}$), 159.0 (*C*=O$_{Phth}$), 166.2 (*C*=O$_{Amide}$). IR (KBr, ν cm$^{-1}$) 1644 (C=O$_{Phth.}$), 3444 (NH). Anal. Calcd for $C_{23}H_{25}N_3O_2$ (375.19): C, 73.57; H, 6.71; N, 11.19 Found C, 73.49; H, 6.64; N, 11.24.

### 5.1.20.6. 2-(4-benzyl-1-oxo-1*H*-phthalazin-2-yl)-*N*-propylacetamide (32c)

Yield: 27.78 % as white crystals, m.p. 130-132°C. $^1$H-NMR (DMSO-$d_6$, 300 MHz) δ 0.87 (t, 3 H, *J* 8 Hz, C*H*$_3$), 1.41-1.49 (m, 2H, C*H*$_2$CH$_3$),3.06-3.11 (m,2H, NHC*H*$_2$), 4.31 (s, 2 H, C*H*$_2$Ph), 4.77 (s, 2 H, NC*H*$_2$), 7.17-8.29 (m, 10 H, Ar*H*+N*H*). $^{13}$C-NMR (DMSO-$d_6$, 75 MHz) δ 11.8 (*C*H$_3$), 22.8 (*C*H$_2$), 38.1 (CH$_2$Ph), 53.6, 53.8 (2 N*C*H$_2$), 126.2, 126.9, 127.0, 128.2, 128.9, 129.0, 129.4, 132.1, 133.0, 1337, 138.6 (12 $C_{aromatic}$), 145.4 (*C*=N$_{Phth.}$), 159.0 (*C*=O$_{Phth.}$), 167.1 (*C*=O$_{Amide}$). IR (KBr, ν cm$^{-1}$) 1647 (C=O$_{Phth.}$), 1661 (*C*=O$_{Amide}$), 3445 (NH). Anal. Calcd for $C_{20}H_{21}N_3O_2$ (335.16): C, 71.62; H, 6.31; N, 12.53 Found C, 71.56; H, 6.45; N, 12.47.

## 5.2. Antitumor activity

### 5.2.1. *In vitro* Anti-Tumor Activity:

Potential cytotoxicity of the newly synthesized compounds was tested against hepatocellular carcinoma cell line (HepG2) using the method of Skehan et al. [30-31]. The in vitro anticancer screening was done by pharmacology unit in cancer biology department at the National Cancer Institute, Cairo University, Cairo, Egypt.

Cells were plated in 96-multiwell plate ($10^4$ cells / well) for 24 hrs. Different concentrations of the compounds under test (0, 5, 12.5, 25, and 50 µg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 hours at 37 $^{o}$C and in atmosphere of 5% $CO_2$. After 48 hrs, cells were fixed, washed and stained with Sulfo-Rhodamine-B stain. Excess stained was washed with acetic acid and attached stained was recovered with Tris EDTA buffer. Colour intensity was measured in an ELISA reader. The relation between surviving fraction and compound concentration is plotted to get the survival curve of tumor cell line after the specified compound.

### 5.2.3. Experimental animals

Swiss albino mice weighing 20 to 25 g were obtained from the Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt) and all animal procedures and experimental protocols were approved by the Research Ethics Committee of Suez Canal University and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

### 5.2.4. Tumor cells

Ehrlich ascites carcinoma (EAC) cell line was purchased from the Tumor Biology Department, National Cancer Institute, Cairo University. EAC is a murine spontaneous breast cancer cells.

### 5.2.5. Induction of solid tumors

Each mouse was inoculated intradermally (i.d.) at 2 sites bilaterally on the lower ventral side with 100 µl EAC suspension ($2.5\times10^6$ cells) on each site [32-33].After treatment, 2-discs/animal, were punched out,

weighed immediately, and the average weight was calculated. All tumors were then kept in 10% neutral buffered formalin for histological evaluation.

### 5.2.6. Pharmacological treatment

Mice were randomly assigned to 4 main groups 8 mice each. The first group received vehicle injection (DMSO in PBS) and served as control. Second and third groups were treated with compounds (10) and (16) (5 mg/kg body weight/ip), while forth group received cisplatin as a positive control, All groups were subjected to a total of seven daily doses during its growth period, starting at the day 6 after inoculation of tumor cells [32-33]. At the end of the experiment, mice were sacrificed by survival dislocation then tumors were excised and fixed in 10% neutral buffered formalin.

### 5.3. Molecular Modelling

All molecular modeling studies were performed on a Hewlett-Packard Pentium Dual-Core T4300 2.10 GHz running Windows 7 Ultimate using autodock 4.3 for molecular docking simulation and ligand binding energy calculation and Molsoft ICM-Pro 3.5-0 for output data visualization. The crystal structure of human EGFR (PDB code; 1xkk) co-crystallized with inhibitor was used as receptors in the docking studies. The selected targets were used after deleting the co-crystallized inhibitors. Docking calculations were carried out using the AutoDock 4.3 software. First all hydrogens were added to the ligand PDB file and Gasteiger charges were computed and all the torsion angles of the ligand were defined with the autodock-tools program, so they could be explored during molecular modeling. A grid box of 25 x 25 x 25Å with a grid spacing of 0.375Å and centered at the crystallized ligand was used to calculate the atom types

needed for the calculation. The Lamarckian genetic algorithm was used as a search method with a total of 30 runs (maximum of 20 000 000energy evaluations; 27 000 generations; initial populations of 150conformers). The docking results were evaluated using binding energy and cluster size calculation in autodock and checking ligand binding position visually in Molsoft ICM-Pro 3.5-0.

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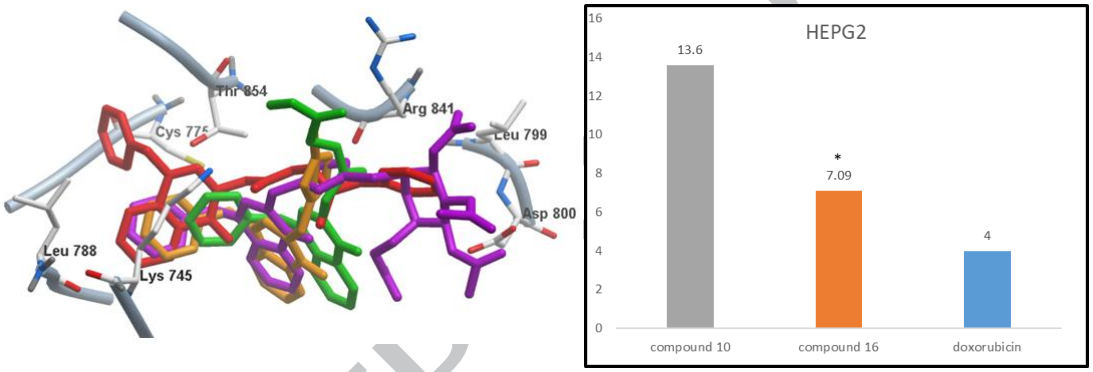
# Design and synthesis of new phthalazine-based derivatives as potential EGFR inhibitors for the treatment of hepatocellular carcinoma

**Ahmed T.A. Boraei, Hanaa K. Ashour, El Sayed H. El Tamany, Nahla Abdelmoaty, Abdullah I. El-Falouji, Mohamed S. Gomaa**




**Novel Potential EGFR inhibitors for the treatment of Hepatocellular Carcinoma**

**Highlights**

- Design and synthesis of phthalazine derivatives
- Characterization of the newly synthesized compounds
- Testing the *in vitro* and *in vivo* antiproliferative activity on HepG2 cancer cell lines
- Using the molecular docking simulation to predict the mode of action