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Original article

Synthesis, molecular modeling and anticancer activity of new coumarin containing compounds

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ABSTRACT

A series of new coumarin containing compounds were synthesized from 4-bromomethylcoumarin derivatives **2a, b** and different heteroaromatic systems **4a-e, 6a-d, 8, 10** *via* methylene thiolinker. Twenty-four compounds were screened biologically against two human tumor cell lines, breast carcinoma MCF-7 and hepatocellular carcinoma HePG-2, at the national cancer institute, Cairo, Egypt using 5-fluorouracil as standard drug. Compounds **5h, 7d, 7**h, **9a, 13a** and **13d** showed strong activity against both MCF-7 and HepG-2 cell lines with being compound **13a** is the most active with IC_{50} values of 5.5 μg/ml and 6.9 μg/ml respectively. Docking was performed with protein 1KE9 to study the binding mode of the designed compounds.

1. Introduction

Uncontrolled cellular proliferation, mediated by dysregulation of the cell-cycle machinery and activation of cyclin-dependent kinases (CDKs) to promote cell-cycle progression, lies at the heart of cancer as a pathological process (O'Leary et al., 2016)

CDKs are key regulatory kinases of the cell cycle (Morgan, 1997). They regulate different phases of cell cycle by binding to distinct regulatory subunit called a cyclin (Abdel Latif et al., 2016). The importance of CDKs in cell division process directed the attention of the medicinal chemists towards the use of them as a potential targets in the treatment of human cancer (Meijer et al., 1999).

Many molecules were found to be CDK specific ATP-competitive inhibitors and few of them have progressed into human clinical trials as flavopiridol compound **I** (Fig. 1) which is a coumarin derivative (Senderowicz et al., 1998 and Sedlacek et al., 1996).

Coumarins are bioactive compounds of both nature and synthetic origin and there has been a growing interest in their synthesis due to their useful and diverse pharmaceutical and biological activities (Salem et al., 2016).

Several heterocyclic compounds containing coumarin ring are associated with diverse pharmacological properties as anti-inflammatory (El-Haggar and Al-Wabli, 2015), antimicrobial (Shi and Zhou, 2011), antiviral (Tsay et al., 2013) and antitumor (Leonetti et al., 2004; Seidel et al., 2014; Jacquot et al., 2007). Moreover, coumarins bearing substitution at 4-position are known to exhibit different biological activities including antiproliferative activity against liver carcinomas ex. compound **II** (Neelgundmath et al., 2015) and compound **III** (Benci et al., 2012) and breast carcinoma as compound **IV** (Bana et al., 2015) and compound **V** (Kini et al., 2012) (Fig. 1). Coumarin itself also exhibited cytotoxic effects against Hep2 cells (human epithelial type 2) in a dose dependent manner and showed some typical characteristics of apoptosis with loss of membrane microvilli, cytoplasmic hypervacualization and nuclear fragmentation (Mirunalini et al., 2014).

Many compounds bearing five-membered heterocyclic rings in their structure have an extensive spectrum of pharmacological activities, among them 1,2,4-triazole,compound **VI** (Park et al., 2009) and **VII** (Reddy et al., 2014),1,3,4-oxadiazole,compound **VIII** (Zhang et al., 2011) and 1,3,4-thiadiazole,compound **IX** (Rashid et al., 2015) have attracted considerable interest as they all showed anticancer activity against various cancer cell lines (Fig. 1).

Based on the previous information we designed and synthesized a series of new coumarin containing compounds where the coumarin ring is substituted at position 4 with different five and

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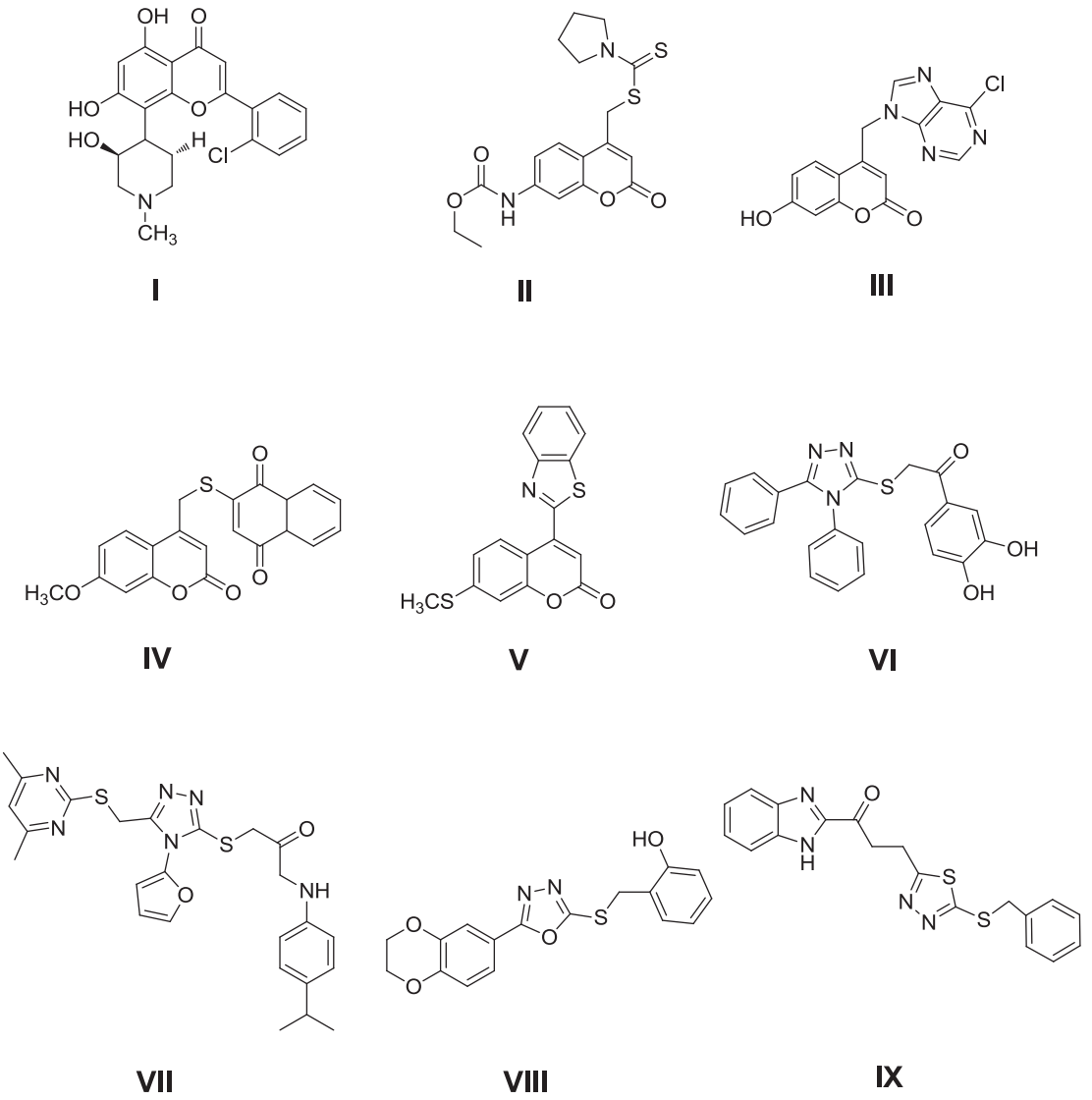


Fig. 1. Structures of anticancer active agents (**I-IX**).

six member heterocycles *via* a methylene thiolinker to test the anticancer effect of these new structure combinations against MCF-7 and HepG-2 cell lines (Fig. 2).

2. Results and discussion

2.1. Chemistry

Scheme 1 illustrates the synthesis of the final compounds **5a-j, 7a-h, 9a, b**. Ethyl 4-bromoacetoacetate **1** was obtained *via* bromination of ethyl acetoacetate using molecular bromine in ether at room temperature (Sousa et al., 2012). This bromo compound was allowed to go through Pechmann condensation with the phenol derivatives **2a, b** in concentrated sulfuric acid to afford the intermediates 4-bromomethylcoumarin derivatives **3a, b** (Khan et al., 2012 and Basanagouda et al., 2014). The triazole derivatives **4a-e** (Kusmeierza et al., 2014; Malbec et al., 1984; Sandstrom and Wennerbeck, 1966) and oxadiazole derivatives **6a-d** (Charistos et al., 1994) were synthesized using the appropriate substituted phenyl hydrazide and phenylisothiocyanate or carbon disulfide, respectively while compound **8** (Kashtoh et al., 2014) was prepared applying the same procedure using hydrazine hydrate and carbon disulfide. The final compounds **5a-j**, **7a-h**, **9a, b** were obtained in good yield after the reaction of the 4-bromomethylcoumarin derivatives **3a, b** with various thiol containing heteroaromatics **4a-e, 6a-d** and **8** in acetone utilizing potassium carbonate as a base

The synthesis of different pyrimidine-5-carbonitril derivatives **13a-d** was described in Scheme 2. The first part of this scheme involved the preparation of the pyrimidine thiol **10** through condensation of benzaldehyde with thiourea and ethyl cyanoacetate according to the reported procedure (Shaquiquzzaman et al., 2012). Alkylation of compound **10** with the bromomethylcoumarin derivative **3a** in tetrahydrofuran using triethylamine as a base afforded the intermediate alkylsulfanyl pyrimidine carbonitrile **11**, which was subsequently halogenated by the reaction with phosphorous oxychloride to give the reactive chloro intermediate **12**. The chloro group in compound **12** was replaced with different aliphatic and aromatic amines in ethanol to afford the final compounds **13a-d** in good yield.

2.2. Antitumor activity

Twenty-four newly synthesized compounds were tested for their cytotoxic activity against human breast cancer cell line (MCF-7) and hepatocellular carcinoma cell line (HepG-2) using

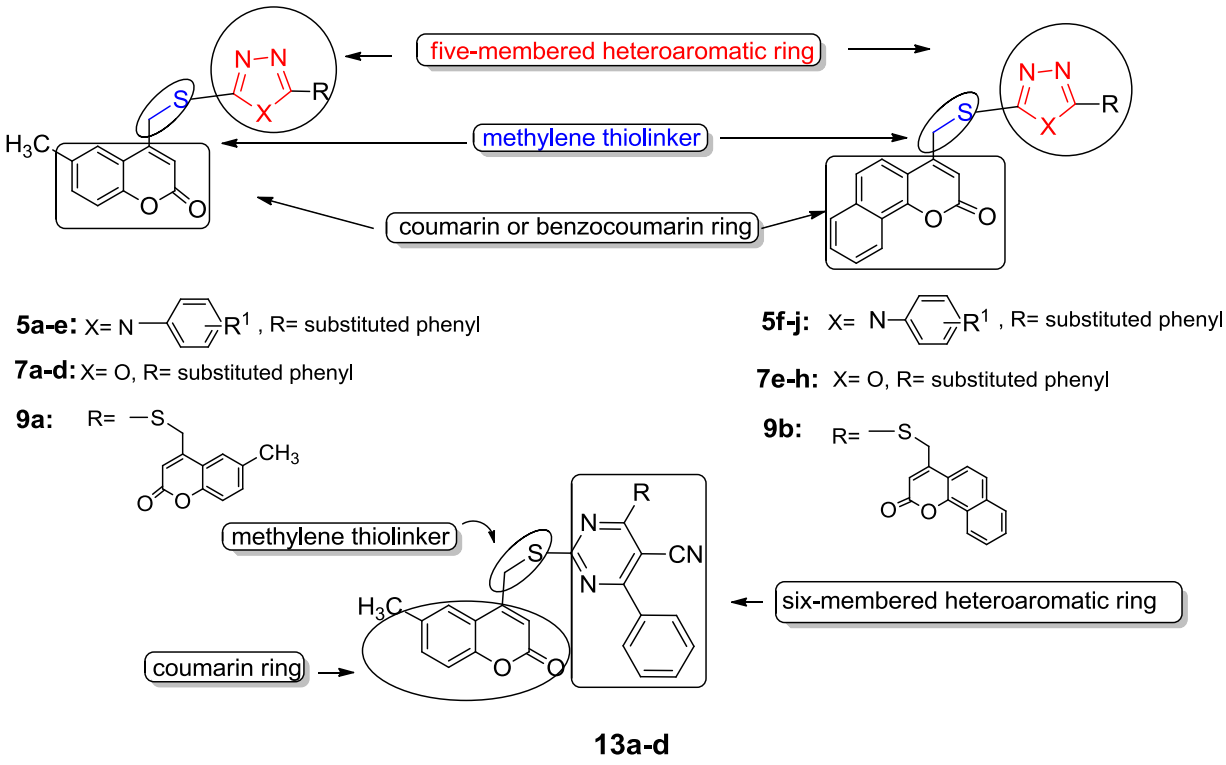


Fig. 2. Structure of the designed target compounds (**5a-j, 7a-h, 9a,b and 13a-d**).

MTT assay according to the method of Mosmann (Mosmann, 1983). The results are presented in Table 1.

A series of 6-methyl-4-substituted coumarin and 4-substituted benzocoumarine was synthesized and evaluated *in vitro* for antitumor activity against MCF-7 and HepG-2 cell line. From the recorded IC_{50}values in Table 1, it was observed that compounds **5h, 7d, 7h, 9a, 13a** and **13d** showed strong activity against both MCF-7 and HepG-2 cell line with being compound **13a** is the most active comparable to 5-Fluorouracil. Compounds **5a, 5c, 5e, 5f, 5i** and **7e** showed moderate activity against both MCF-7 and HepG-2 cell lines. Compounds **7b** and **7c** showed moderate activity against MCF-7 and weak activity against HepG-2 cell line. Compounds **5b, 5j, 5g, 7f, 7g**, **9b, 13b** and **13c** showed weak activity against both MCF-7 and HepG-2 cell lines.

From the above data we conclude that the substitution of the nitro group of **5h** alters the activity either with methyl group leads to almost inactive compound **5g, 5j**, while replacing it with hydrogen decreases the activity as in compounds **5f** and **5i.** Moreover replacing the benzocoumarine ring of **5h** with methylcoumarine ring reduces the activity as in compound **5c.** Moving to the oxadiazole series **7a-7h** we realized that having fluorine substituted phenyl ring attached to the oxadiazole moiety leads to the most active compounds in these series either with methylcoumarine moiety attached to the oxadiazole ring **7d** or with benzocoumarine ring attached to the oxadiazole ring **7h**. Replacing this fluorine atom in this series with any other substituents either reduce (**7b, 7c,** and **7e**) or kills the activity (**7a, 7f** and **7g**). Having disubstituted thiadiazole with methylcoumarine compound gives strong active compound **9a**, while replacing these two methylcoumarine rings with benzocoumarine ones leads to inactive compound **9b**.

The replacement of the hydrazinyl moiety or the piperazinyl one on the pyrimidine ring in **13a** and **13d** by aniline in **13b** or morpholine in **13c** respectively resulted in reducing or killing the activity concluding that the most active compound should bear a hydrogen bond donor moiety like in **13 a, 13d**.

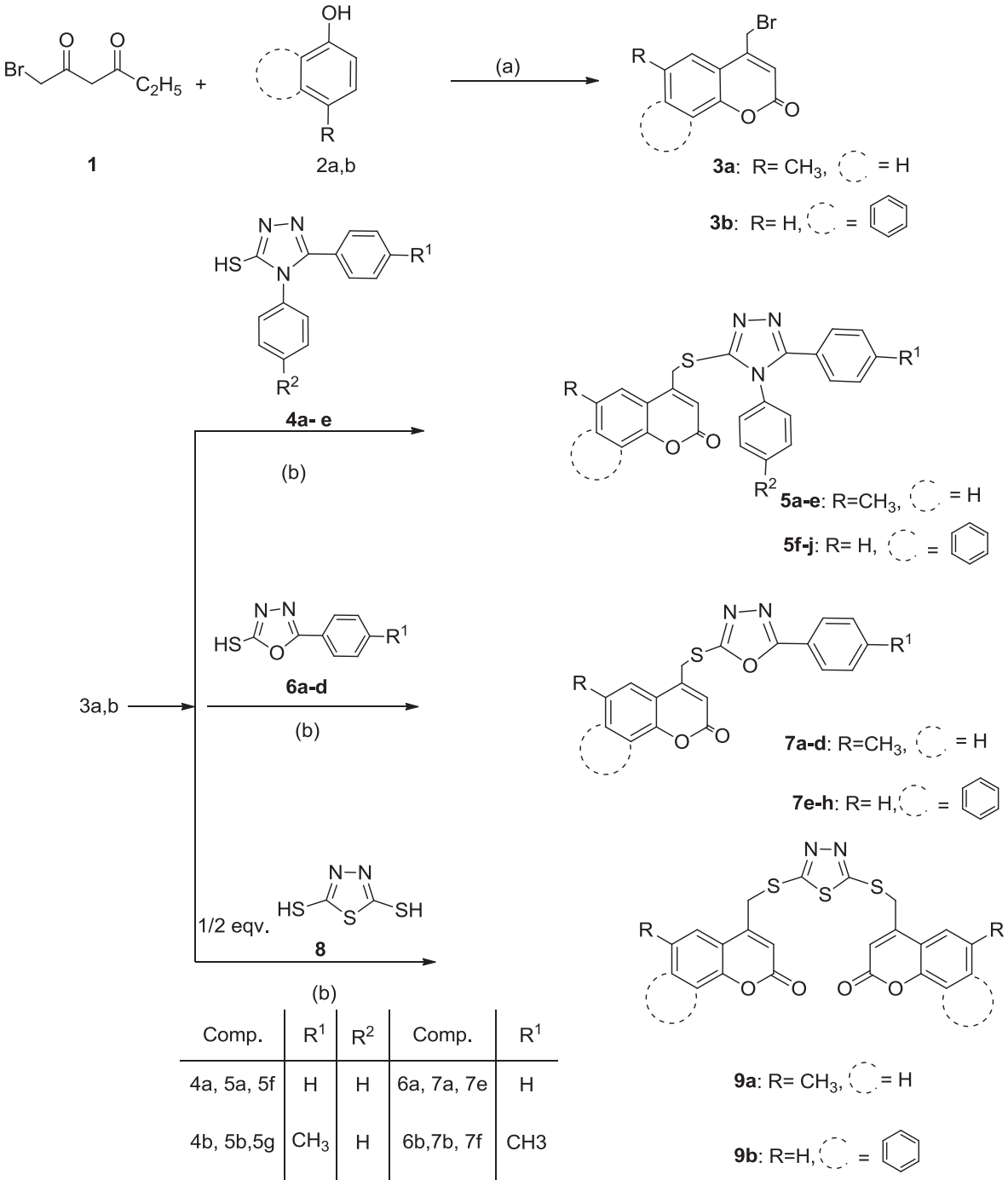
2.3. Molecular docking

Molecular docking is a computational procedure performed on structure-based rational drug design to identify correct conformations of small molecule ligands and also to estimate the strength of the protein-ligand interaction, usually one receptor and one ligand. Docking of ligands that are bound to a receptor through non-covalent interactions is relatively conventional nowadays. The majority of docking methods development research has been focused on the effective prediction of the binding modes of non-covalent inhibitors (Kumalo et al., 2015).

CDKs have a critical role in the control of cell division so deregulation of CDKs can lead to abnormal processes and numerous human diseases, most notably cancer and tumors. Therefore, suppression of CDKs activity serves as an ideal therapeutic strategy for cancer and tumor by interrupting aberrant cell proliferation (Lu et al., 2016). A new class of drugs, termed CDK inhibitors, has been studied in preclinical and now clinical trials. These inhibitors are believed to act as an anti-cancer drug by blocking CDKs to block the uncontrolled cellular proliferation that is hallmark of cancers (Balakrishnan et al., 2016)

Docking Studies for the target compounds **5h, 9a and 13a**, which showed the highest *in vitro* activity, were carried out using the molecular operating environment (MOE) to show their binding mode and suggest the proposed mechanism of their antiproliferative activity. Crystallographic structure of cyclin dependent kinase 2 (CDK2) was obtained from the protein data bank (code, 1KE9. pdb) (Bramson et al., 2001) with the help of Pharm Mapper software (Liu et al., 2010). Upon inspection of the protein embedded ligand interaction for the protein molecule 1KE9, it was found that the embedded ligand occupied the ATP-binding site of CDK2 with the formation of two hydrogen bonds, one with the backbone NH of Leu-83 and the other with the backbone carbonyl of Glu-81 as shown in (Fig. 3).

When examining the interaction between the protein and our target compounds, it was found that the target compounds



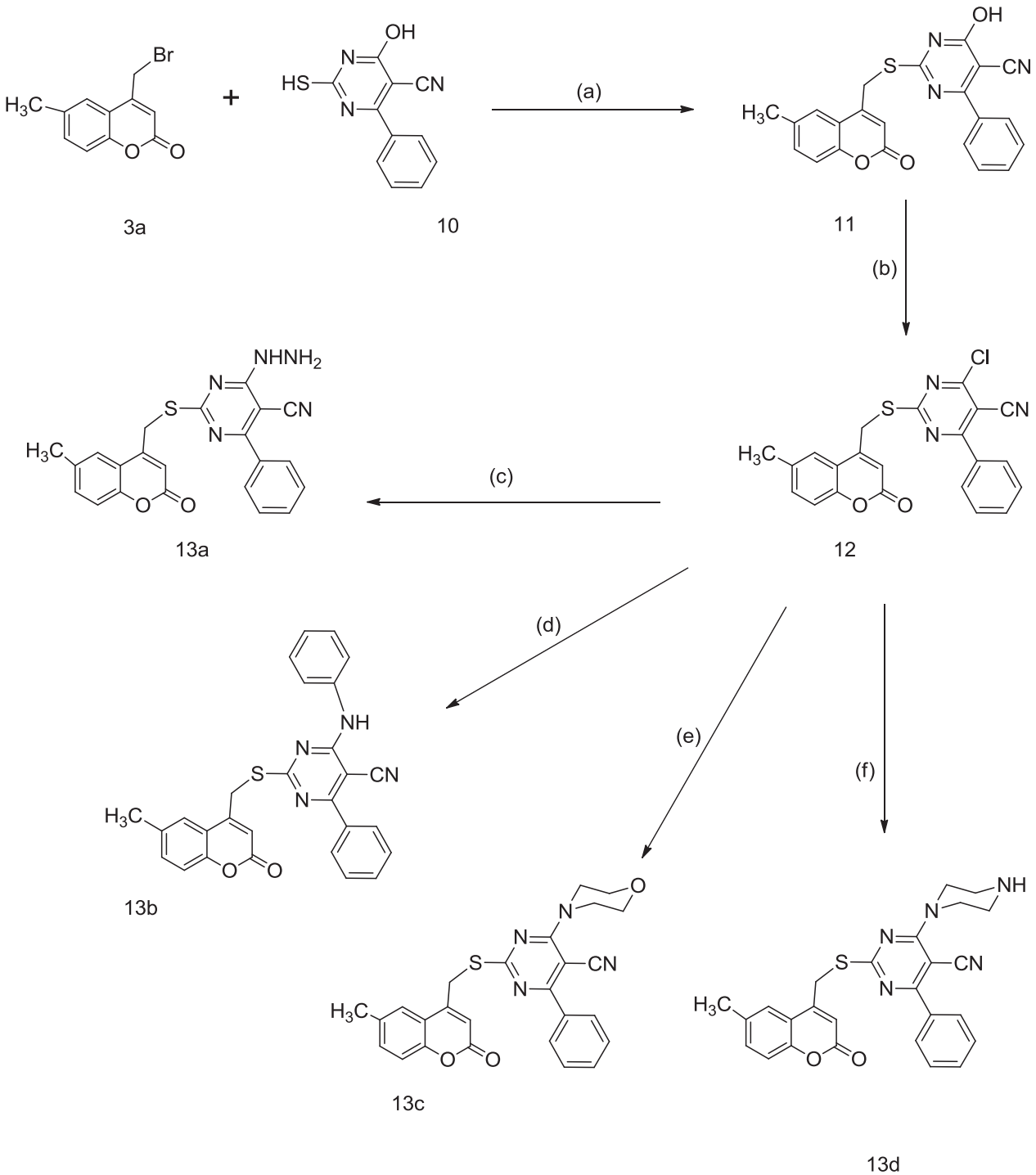
Comp.	R^1	R^2	Comp.	R^1
4a, 5a, 5f	H	H	6a, 7a, 7e	H
4b, 5b,5g	CH_3	H	6b,7b, 7f	CH3
4c, 5c, 5h	NO_2	H	6c,7c, 7g	NO2
4d, 5d, 5i	H	F	6d, 7d, 7h	F
4e, 5e, 5j	CH_3	F		

Scheme 1. Reagents and conditions: (a) H_2SO_4. 0 °C, (b) K_2CO_3, Acetone, r.t.

occupied the same pocket of the active site of the protein and formed one hydrogen bond with backbone NH of Leu-83 as shown in (Figs. 4–6). These interactions assume the importance of Leu-83 residue for the activity. Also it was observed that compound **5h** showed an additional binding to Lys-89 through arene cation interaction, but this interaction does not seem to have any relation to activity.

3. Conclusion

The target compounds showed good fit within the active site of the docked CDK2. There is a strong correlation between molecular modeling and biological screening results and it is expected that the target compounds antiproliferative activity may be due to inhibition of CDK2 activity.



Scheme 2. Reagents and conditions: (a) THF, TEA, r.t., (b) $POCl_3$, reflux, (c) NH_2NH_2, EtOH, r.t., (d) Aniline, EtOH, TEA, reflux. (e) Morpholine, EtOH, K_2CO_3, reflux. (f) Piperazine, EtOH, K_2CO_3, reflux.

4. Experimental

4.1. Chemistry

All commercial reagents were used without purification. Melting points were determined on a Mel-Temp 3.0 melting point apparatus, and are uncorrected. TLC analysis was carried out on silica gel 60 F254 precoated aluminum sheets using UV light for detection. ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer and Bruker 100 MHz spectrometer, respectively using the indicated solvents. Mass spectra were obtained from the Cairo University Mass Spectrometry Laboratory, Cairo, Egypt. Elemental analysis was performed at the Microanalysis Centre, Cairo University.

4.1.1. Synthesis of ethyl 4-bromoacetoacetate (**1**)

The required ethyl 4-bromoacetoacetate was synthesized by bromination of ethyl acetoacetate with molecular bromine in dry ether according to the published method (Sousa et al., 2012).

Table 1
Cytotoxic activity against two human tumor cell lines.

Compounds	*In vitro* Cytotoxicity IC_{50} (μg/ml)	
	HePG-2	MCF-7
5-FU	7.9 ± 0.17	5.4 ± 0.20
5a	32.7 ± 2.84	47.7 ± 2.89
5b	57.9 ± 3.87	63.5 ± 4.23
5c	26.9 ± 2.11	22.1 ± 1.84
5d	62.8 ± 4.26	69.2 ± 4.56
5e	41.5 ± 3.02	29.7 ± 1.95
5f	20.3 ± 1.89	13.2 ± 1.12
5g	96.4 ± 5.40	83.8 ± 5.12
5h	12.5 ± 0.97	9.0 ± 0.81
5i	45.2 ± 3.28	40.5 ± 2.46
5j	76.9 ± 4.82	58.7 ± 3.72
7a	100<	100<
7b	58.1 ± 4.13	21.1 ± 1.63
7c	67.7 ± 4.32	39.7 ± 2.54
7d	13.6 ± 1.06	16.0 ± 1.38
7e	31.1 ± 2.45	33.6 ± 2.37
7f	73.0 ± 4.71	51.0 ± 3.45
7g	84.4 ± 4.96	81.5 ± 4.95
7h	18.2 ± 1.57	19.8 ± 1.40
9a	8.3 ± 0.25	7.7 ± 0.56
9b	86.0 ± 5.15	77.9 ± 4.81
13a	5.5 ± 0.19	6.9 ± 0.38
13b	52.0 ± 3.55	49.0 ± 3.10
13c	91.1 ± 5.27	100<
13d	15.8 ± 1.23	10.9 ± 0.97

5-FU = 5-fluorouracil.

*4.1.2. Synthesis of 4-(bromomethyl)-6-methyl-2H-chromen-2-one (**3a**)*

It was synthesized according to the procedure described by (Khan et al., 2012).

*4.1.3. Synthesis of 4-(bromomethyl)-2H-naphtho[1, 2-b]pyran-2-one (**3b**)*

It was synthesized using known procedure (Basanagouda et al., 2014).

*4.1.4. General procedure for the preparation of compounds (**4a-c** and **6a-d**)*

The required 4*H*-1,2,4-triazole-3-thiol derivatives (**4a-c**) was synthesized according to the published procedure (Sandstrom and Wennerbeck, 1966) while 1,3,4-oxadiazole-2-thiol derivatives (**6a-c**) was synthesized using the known procedure (Charistos et al., 1994).

*4.1.5. General procedure for the preparation of compounds (**4d-e**)*

Potassium hydroxide solution (1.4 gm, 0.025 mol) in ethanol (100 ml) was added to the appropriate benzohydrazide (0.025 mol) under stirring. After a few minutes 4-fluorophenylisothiocyanate (3.82 gm, 0.025 mol) was added and the mixture was refluxed for 8 h. The solvent was concentrated under reduced pressure. Ice water was added to the residue and acidified by hydrochloric acid to give white solid which was filtered off, dried and recrystallized from a mixture of ethanol-water (1:1).

*4.1.6. 4-(4-Fluorophenyl)-5-phenyl-4H-1,2,4-triazole-3-thiol (**4d**)*

White solid, yield 75%, m p 186–188 °C: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.25–7.39 (m, 5H), 7.40–7.70 (m, 4H), 10.60 (s, 1H, D_2O-exchangeable); ESI-MS: *m/z* calculated for $C_{14}H_{10}FN_3S$: 271.1, found: 272.5 (M^++1); Anal. Calcd. For $C_{14}H_{10}FN_3S$: C, 61.98; H, 3.72; N, 15.49. Found: C, 62.12; H, 3.54; N, 15.65.

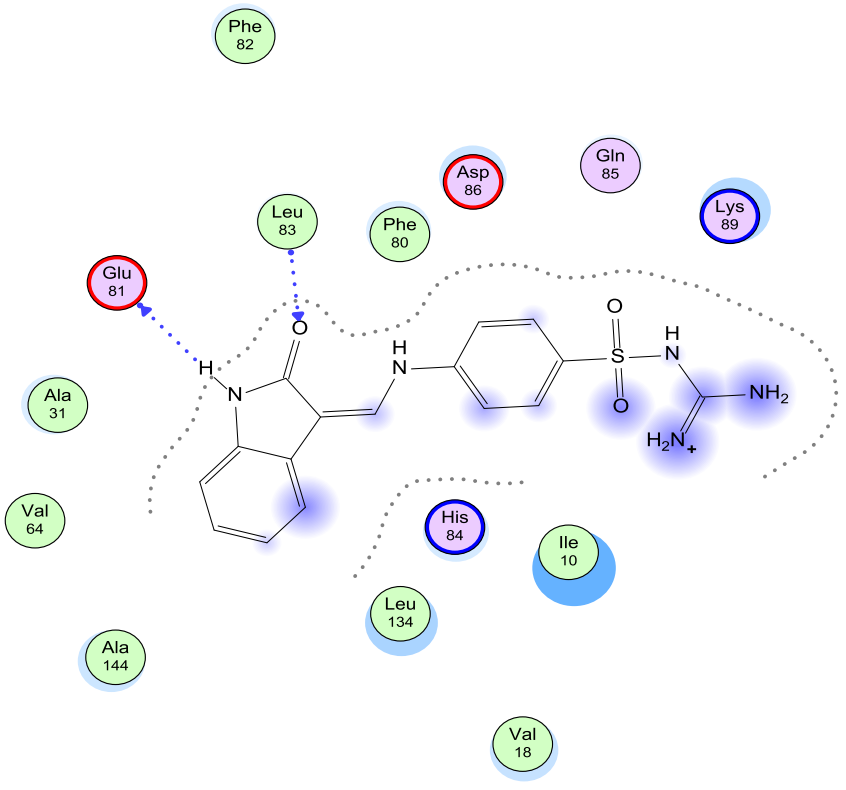


Fig. 3. Binding mode of the embedded ligand within 1KE9 active site.

*4.1.7. 4-(4-Fluorophenyl)-5-(4-methylphenyl)-4H-1,2,4-triazole-3-thiol (**4e**)*

White solid, yield 78%, m p 222–224 °C: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.27 (s, 3H), 7.15–7.21 (m, 4H), 7.31–7.40 (m, 2H), 7.41–7.44 (m, 2H), 10.57 (s, 1H, D_2O-exchangeable); ESI-MS: *m/z* calculated for $C_{15}H_{12}FN_3S$: 285.1, found: 286.5 (M^++1); Anal. Calcd. For $C_{15}H_{12}FN_3S$: C, 63.14; H, 4.24; N, 14.73. Found: C, 63.22; H, 3.35; N, 14.55.

*4.1.8. General procedure for the preparation of compounds (**5a-j** and **7a-h**)*

A mixture of **4a-e** or **6a-d** (0.005 mol) and anhydrous potassium carbonate (0.69 gm, 0.005 mol) was stirred for 0.5 h in dry acetone (10 ml). **3a** or **3b** (0.005 mol) was added to the reaction mixture and the stirring was continued for 24 h at room temperature. Ice (10 gm) was added to the reaction mixture and the separated solid was filtered then recrystallized from acetone.

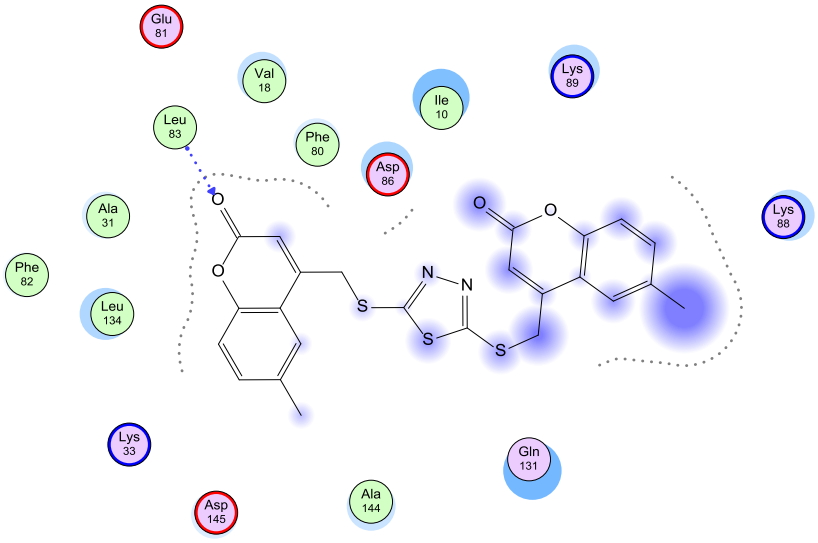


Fig. 5. Docking of **9a** in 1KE9 active site.

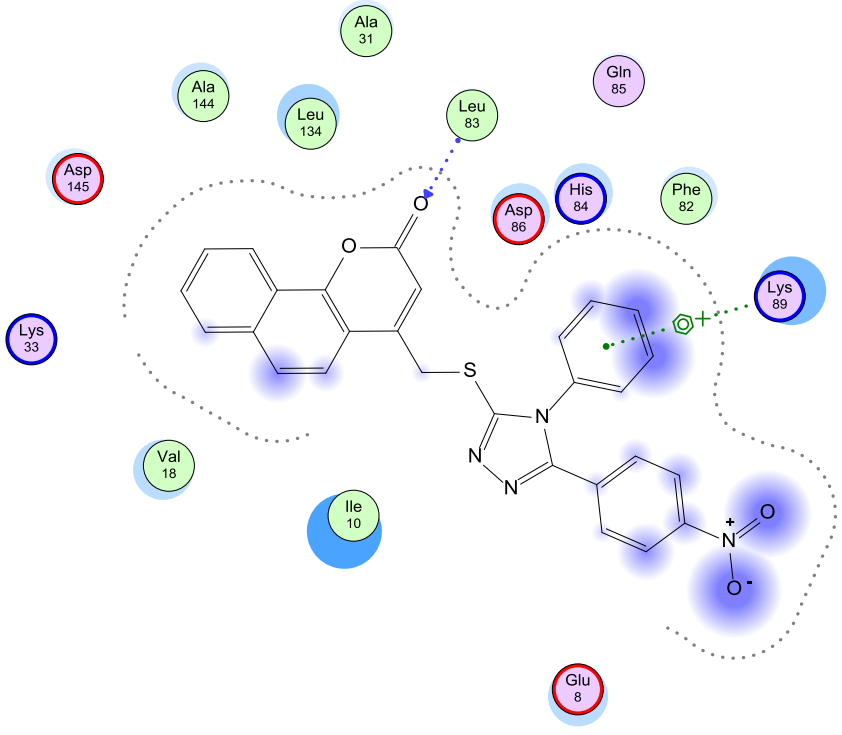


Fig. 4. Docking of **5h** in 1KE9 active site.

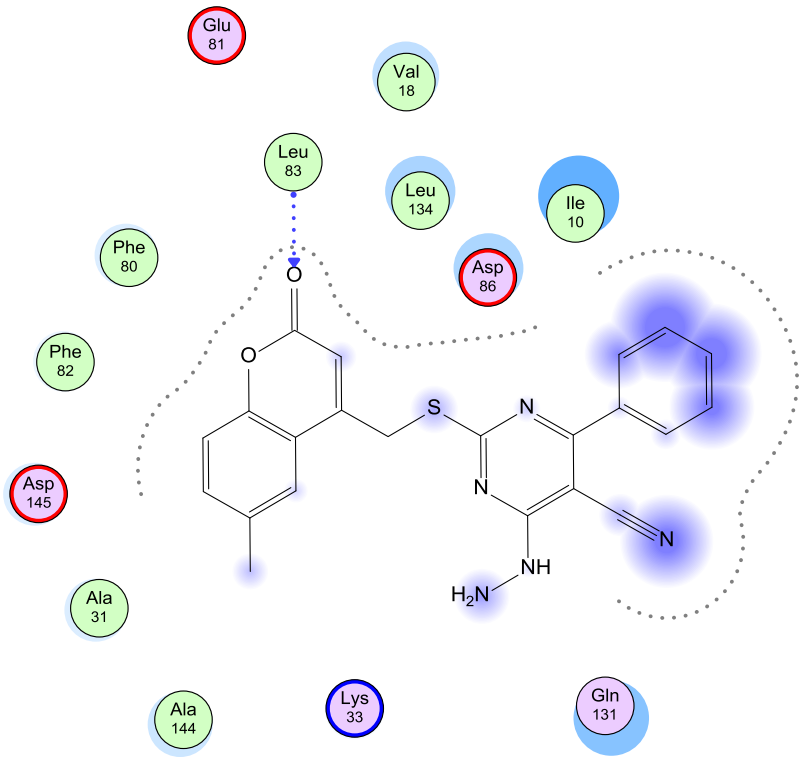


Fig. 6. Docking of **13a** in 1KE9 active site.

*4.1.9. 4-{[(4,5-Diphenyl-4H-1,2,4-triazol-3-yl)sulfanyl]methyl}-6-methyl-2H-chromen-2-one (**5a**)*

Brownish white solid, Yield (1.69 gm, 80%), mp 166–168 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.35 (s, 3H), 4.56 (s, 2H), 6.50 (s, 1H), 7.29–7.38 (m, 8H), 7.43–7.48 (m, 4H), 7.63 (s, 1H); ESI-MS: *m/z* calculated for $C_{25}H_{20}N_3O_2S$: 425.1, found: 426 (M^++1); Anal. Calcd. For $C_{25}H_{20}N_3O_2S$: C, 70.57; H, 4.50; N, 9.88. Found: C, 70.73; H, 4.69; N, 9.75.

*4.1.10. 6-Methyl-4-({[4-phenyl-5-(4-methylphenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}methyl)-2H-chromen-2-one (**5b**)*

Brownish white solid, Yield (1.79 gm, 82%), mp 183–185 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.26 (s, 3H), 2.35 (s, 3H), 4.45(s, 2H), 6.49(s, 1H), 7.14–7.28(m, 7H), 7.46(br s, 4H), 7.62 (s, 1H); ESI-MS: *m/z* calculated for $C_{26}H_{22}N_3O_2S$: 439.1, found:440 (M^++1); Anal. Calcd. For $C_{26}H_{22}N_3O_2S$: C, 71.05; H, 4.82; N, 9.56. Found: C, 71.25; H, 5.00; N, 9.34.

*4.1.11. 6-Methyl-4-({[5-(4-nitrophenyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio}sulfanyl)-2H-chromen-2-one (**5c**)*

Brownish yellow solid, Yield (1.66 gm, 71%), mp 190–192 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.35 (s, 3H), 4.61 (s, 2H), 6.53 (s, 1H), 7.32–7.65 (m, 10H), 8.19 (br s, 2H); ESI-MS: *m/z* calculated for $C_{25}H_{19}N_4O_4S$: 470.1, found: 471.95 (M^++1); Anal. Calcd. For $C_{25}H_{19}N_4O_4S$: C, 63.82; H, 3.86; N, 11.91. Found: C, 63.68; H, 3.96; N, 11.77.

*4.1.12. 4-({[4-(4-Fluorophenyl)-5-(phenyl)-4H-1,2,4-triazol-3-yl]sulfanyl} methyl)-6-methyl-2H-chromen-2-one (**5d**)*

White solid, Yield (1.66 gm, 75%) mp 184–186 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.35 (s, 3H), 4.56 (s, 2H), 6.50 (s, 1H), 7.34 (br s, 8H), 7.47 (br s, 3H), 7.63 (s, 1H); ESI-MS: *m/z* calculated for $C_{25}H_{19}FN_3O_2S$: 443.1, found: 444.98 (M^++1); Anal. Calcd. For $C_{25}H_{19}$ FN_3O_2S: C, 67.71; H, 4.09; N, 9.47. Found: C, 67.84; H, 4.27; N, 9.23.

*4.1.13. 4-({[4-(4-Fluorophenyl)-5-(4-methylphenyl)-4H-1,2,4-triazol-3-yl] sulfanyl}methyl)-6-methyl-2H-chromen-2-one (**5e**)*

White solid, Yield (1.78 gm, 78%), mp 198–200 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.27 (s, 3H), 2.35 (s, 3H), 4.52 (s, 2H), 6.48 (s,1H), 7.03–7.26 (m, 5H), 7.27–7.33 (br s, 2H), 7.34–7.42 (br s, 2H), 7.43–7.44 (m, 1H), 7.62 (s, 1H); ESI-MS: *m/z* calculated for $C_{26}H_{21}FN_3O_2S$: 457.1, found: 458 (M^++1); Anal. Calcd. For $C_{26}H_{21}$-FN_3O_2S: C, 68.25; H, 4.41; N, 9.18. Found: C, 68.34; H, 4.23; N, 9.03.

*4.1.14. 4-{[(4,5-Diphenyl-4H-1,2,4-triazol-3-yl)sulfanyl]methyl}-2H-benzo [h]-2-one (**5f**)*

White solid, Yield (1.70 gm, 74%), mp 160–162 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.66 (s, 2H), 6.61 (s, 1H0, 7.34 (br s, 4H), 7.39 (br s, 4H), 7.73 (br s, 4H), 7.83 (br s, 2H), 8.03–8.05 (d, 1H, J = 8 Hz), 8.36–8.38 (d, 1H, J = 6);ESI-MS: *m/z* calculated for $C_{28}H_{20}$-N_3O_2S: 461.1, found: 462 (M^++1); Anal. Calcd. For $C_{28}H_{20}N_3O_2S$: C, 72.87; H, 4.15; N, 9.10. Found: C, 72.71; H, 4.24; N, 9.25.

*4.1.15. 4-({[5-(4-Methylphenyl)-4-phenyl-4H-1,2,4-triazol-3-yl]sulfanyl} methyl)-2H-benzo[h]chromen-2-one (**5g**)*

White solid, Yield (1.80 gm, 76%), mp 180–182 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ:2.27 (s, 3H), 4.65 (s, 2H), 6.58 (s, 1H), 7.12–7.14 (m, 2H), 7.23–7.27 (m, 4H), 7.39–7.40 (m, 3H), 7.71–7.73 (m, 2H), 7.80 (brs, 2H), 8.02–8.04 (m, 1H), 8.37–8.40 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6, 32.8, 119.4, 120.4, 122.6, 123.2, 123.9, 124.1, 124.6, 126.7, 126.8, 127.1, 127.4, 127.7, 128.6, 129.0, 129.1, 129.8, 131.9, 134.9, 142.5, 149.5, 149.6, 151.3, 160.1; ESI-MS: *m/z* calculated for $C_{29}H_{22}N_3O_2S$: 475.1,found: 475.95 (M^++1); Anal. Calcd. For $C_{29}H_{22}N_3O_2S$: C, 73.24; H, 4.45; N, 8.84. Found: C, 73.05; H, 4.71; N, 8.61.

*4.1.16. 4-({[5-(4-Nitrophenyl)-4-phenyl-4H-1,2,4-triazol-3-yl]sulfanyl} methyl)-2H-benzo[h]chromen-2-one (**5h**)*

Brownish yellow solid, Yield (1.87 gm, 74%), mp 171–173 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 4.71 (s, 2H), 6.65 (s, 1H), 7.35–7.48 (m,

7H), 7.59 (br s, 2H), 7.72 (br s, 1H), 7.84 (br s, 2H), 8.18 (br s, 2H), 8.30 (br s, 1H); ESI-MS: *m/z* calculated for $C_{28}H_{19}N_4O_4S$: 506.1, found: 507.90 (M^++1); Anal. Calcd. For $C_{28}H_{19}N_4O_4S$: C, 66.39; H, 3.58; N, 11.06. Found: C, 66.46; H, 3.32; N, 11.25.

*4.1.17. 4-({[4-(4-Fluorophenyl)-5-phenyl-4H-1,2,4-triazol-3-yl]sulfanyl} methyl)-2H-benzo[h]chromen-2-one (**5i**)*

Greyish white solid, Yield (1.62 gm, 68%), mp 194–196 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.64 (s, 2H), 6.60 (s, 1H), 7.20–7.24 (m, 2H), 7.35–7.40 (m, 6H), 7.72–7.73 (br s, 3H), 7.83 (br s, 2H), 8.03–8.04 (br s, 1H), 8.35–8.37 (br s, 1H); ESI-MS: *m/z* calculated for $C_{28}H_{19}FN_3O_2S$: 479.1, found: 480 (M^++1); Anal. Calcd. For $C_{28}H_{19}FN_3O_2S$: C, 70.13; H, 3.78; N, 8.76. Found: C, 70.02; H, 4.01; N, 8.83.

*4.1.18. 4-({[4-(4-Fluorophenyl)-5-(4-methylphenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}methyl)-2H-benzo[h]chromen-2-one (**5j**)*

Yellow solid, Yield (1.72 gm, 70%), mp 139–141 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ:2.26 (s, 3H), 4.62 (s, 2H), 6.59 (s, 1H), 7.15–7.17 (m, 2H), 7.19–7.23 (m, 4H), 7.37–7.40 (m, 2H), 7.72–7.74 (m, 2H), 7.83 (br s, 2H), 8.03–8.05 (d, 1H, J = 7 Hz), 8.36–8.37 (d,1H, J = 6 Hz); ESI-MS: *m/z* calculated for $C_{29}H_{21}FN_3O_2S$: 493.1, found: 494 (M^++1); Anal. Calcd. For $C_{29}H_{21}FN_3O_2S$: C, 70.57; H, 4.08; N, 8.51. Found: C, 70.61; H, 3.93; N, 8.59.

*4.1.19. 6-Methyl-4-{[(5-phenyl-1,3,4-oxadiazol-2-yl)sulfanyl] methyl}-2H-chromen-2-one (**7a**)*

Greyish white solid, Yield (1.33 gm, 76%), mp 150–152 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.38 (s, 3H), 4.77 (s, 2H), 6.60 (s, 1H), 7.12–7.53 (m, 4H), 7.80 (br s, 1H), 7.82 (br s, 2H), 7.94 (s, 1H); ESI-MS: *m/z* calculated for $C_{19}H_{15}N_2O_3S$: 350.1, found: 351 (M^++1); Anal. Calcd. For $C_{19}H_{15}N_2O_3S$: C, 65.13; H, 4.03; N, 7.99. Found: C, 65.21; H, 4.10; N, 7.84.

*4.1.20. 6-Methyl-4-({[(5-methylphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl}methyl) -2H-chromen-2-one (**7b**)*

Brownish white solid, Yield (1.43 gm, 79%), mp 174–176 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ:2.39 (s, 6H), 4.70 (s, 2H), 6.60 (s, 1H), 7.39–7.46 (m, 4H), 7.83 (br s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.0, 21.6, 32.3, 116.4, 117.3, 117.5, 120.5, 123.8, 126.7, 129.8, 133.3, 142.5, 148.5, 152.0, 160.6, 161.98, 166.6; ESI-MS: *m/z* calculated for $C_{20}H_{17}N_2O_3S$: 364.1, found: 365.97 (M^++1); Anal. Calcd. For $C_{20}H_{17}N_2O_3S$: C, 65.92; H, 4.43; N, 7.69. Found: C, 65.99; H, 4.37; N, 7.72.

*4.1.21. 6-Methyl-4-({[5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl]sulfantl} methyl) -2H-chromen-2-one (**7c**)*

Brownish yellow solid, Yield (1.38 gm, 70%) mp 190–192 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ:2.39(s, 3H),4.82(s, 2H), 6.64 (s,1H), 7.33 (br s,1H), 7.46 (br s,1H), 7.84 (s, 1H), 8.21 (br s, 2H), 8.38 (br s, 2H);ESI-MS: *m/z* calculated for $C_{19}H_{14}N_3O_5S$: 395.1, found: 396.90 (M^++1); Anal. Calcd. For $C_{19}H_{14}N_3O_5S$: C, 57.72; H, 3.31; N, 10.63. Found: C, 57.61; H, 3.39; N, 10.54.

*4.1.22. 4-({[5-(4-Fluorophenyl)-1,3,4-oxadiazol-2-yl]sulfanyl} methyl)-6-methyl-2H-chromen-2-one (**7d**)*

Grey solid, Yield (1.28 gm, 70%), mp 141–143 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.39 (s,3H), 4.82 (s, 2H), 6.64 (s,1H), 7.33 (bs,1H), 7.46 (bs, 1H), 7.84 (s, 1H), 8.20 (bs, 2H), 8.39 (bs, 2H); ESI-MS: *m/z* calculated for $C_{19}H_{14}N_3O_5S$: 368.1, found: 369 (M^++1); Anal. Calcd. For $C_{19}H_{14}N_3O_5S$: C, 61.95; H, 3.56; N, 7.60. Found: C, 61.77; H, 3.60; N, 7.74.

*4.1.23. 4-{[(5-Phenyl-1,3,4-oxadiazol-2-yl)sulfanyl]methyl}-2H-benzo [h] chromen-2-one (**7e**)*

white solid, Yield (1.44 gm, 75%), mp 150–152 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.98 (s, 2H), 6.72–6.73 (br s,1H), 7.34–7.36 (d, 1H, J = 7.6 Hz), 7.55–7.61 (m, 2H), 7.71 (br s, 2H), 7.80–7.81 (d, 1H, J = 7.6 Hz), 7.88–7.93 (m, 2H), 8.01–8.03 (m, 2H), 8.33–8.35 (d, 1H, J = 8.8 Hz);ESI-MS: *m/z* calculated for $C_{22}H_{15}N_2O_3S$: 386.1, found: 386.97 (M^++1); Anal. Calcd. For $C_{22}H_{15}N_2O_3S$: C, 68.38; H, 3.65; N, 7.25. Found: C, 68.44; H, 3.41; N, 7.33.

*4.1.24. 4-({[5-(4-Methylphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl} methyl)-2H-benzo[h]chromen-2-one (**7f**)*

white solid, Yield (1.54 gm, 77%), mp 177–178 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.39 (s, 3H), 4.88 (s, 2H), 6.72 (s, 1H), 7.35–7.37 (d, 2H, J = 7.6 Hz), 7.71–7.73 (m, 2H), 7.80–7.82 (d, 2H, J = 7.6 Hz), 7.87–7.90 (d, 1H, J = 12.8 Hz), 7.99–8.01 (d, 1H, J = 8.8 Hz), 8.03–8.05 (m, 1H), 8.37–8.39 (d, 1H, J = 8 Hz,); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.3, 33.3, 119.8, 122.6, 123.2, 123.3, 124.3, 127.1, 127.6, 128.0, 128.8, 129.2, 129.9, 133.8, 134.8, 140.2, 150.5, 150.7, 151.1, 155.6, 160.3; ESI-MS: *m/z* calculated for $C_{23}H_{17}N_2O_3S$: 400.1, found: 401 (M^++1); Anal. Calcd. For $C_{23}H_{17}N_2O_3S$: C, 68.98; H, 4.03; N, 7.00. Found: C, 68.89; H, 4.15; N, 6.94.

*4.1.25. 4-({[5-(4-Nitrophenyl)-1,3,4-oxadiazol-2-yl]sulfanyl}methyl)-2H-benzo[h]chromen-2-one (**7g**)*

Brown solid, Yield (1.50 gm, 70%), mp 200–202 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.94 (s, 2H), 6.78 (s, 1H), 7.63–7.80 (m, 1H), 7.94 (m, 1H), 8.06 (m, 2H), 8.19–8.21 (m, 2H), 8.37 (m, 4H); ESI-MS: *m/z* calculated for $C_{22}H_{14}N_3O_5S$: 431.1, found: 432.95 (M^++1); Anal. Calcd. For $C_{22}H_{14}N_3O_5S$: C, 61.25; H, 3.04; N, 9.74. Found: C, 61.33; H, 2.98; N, 9.66.

*4.1.26. 4-({[5-(4-Fluorophenyl)-1,3,4-oxadiazol-2-yl]sulfanyl} methyl)-2H-benzo[h]chromen-2-one (**7h**)*

Yellowish white solid, Yield (1.41 gm, 70%), mp 156–158 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.93 (s, 2H), 6.78 (s,1H), 7.65–7.80 (m, 3H), 7.92 (m,1H), 8.02–8.05 (m, 2H), 8.20–8.26 (m, 1H), 8.30–8.39 (m, 3H);ESI-MS: *m/z* calculated for $C_{22}H_{14}FN_2O_3S$:404.1, found: 405.90 (M^++1); Anal. Calcd. For $C_{22}H_{14}FN_2O_3S$: C, 65.34; H, 3.24; N, 6.93. Found: C, 65.23; H, 3.29; N, 6.77.

*4.1.27. Synthesis of 1,3,4-thiadiazole-2,5-dithiol (**8**)*

Compound **8** was synthesized according to the published method (Kashtoh et al., 2014).

*4.1.28. Synthesis of 4,4′-{[(1,3,4-thiadiazole-2,5-diyl)bis(sulfanediyl)] bis (methylene)}bis(6-methyl-2H-chromen-2-one)(9a)and4,4′-{[(1,3,4-thiadiazole-2,5-diyl)bis(sulfanediyl)]bis(methylene)}bis(2H-benzo[h] chromen-2-one) (**9b**)*

A mixture of 1,3,4-thiadiazole-2,5-dithiol **(8)** (0.75gm, 0.005 mol) and anhydrous potassium carbonate (1.38 gm, 0.01 mol) was stirred for 1 h in dry acetone (10 ml) at room temperature. **3a** or **3b**(0.01 mol) was added and the stirring was continued for 24 h at room temperature. Then the resulting reaction mixture was poured into crushed ice. The separated solid was filtered, air dried and recrystallized from dimethylformamide.

*4.1.29. 4,4′-{[(1,3,4-Thiadiazole-2,5-diyl)bis(sulfanediyl)]bis (methylene)}bis (6-methyl-2H-chromen-2-one) (**9a**)*

Yellow white solid, Yield (1.5 gm, 62%), mp 131–133 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.37 (s, 6H), 4.70 (s,4H), 6.50 (s, 2H), 7.31 (br s, 2H), 7.46 (br s, 2H), 7.76 (br s, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz)δ 20.9, 34.3, 115.3, 116.9, 117.8, 125.6, 125.7, 133.4, 134.1, 151.7, 151.9, 160.1;ESI-MS: *m/z* calculated for $C_{24}H_{18}N_2O_4S_3$: 494, found: 495.80 (M^++1); Anal. Calcd. For C_{24}-

$H_{18}N_2O_4S_3$: C, 58.28; H, 3.67; N, 5.66. Found: C, 58.46; H, 3.85; N, 5.36.

*4.1.30. 4,4′-{[(1,3,4-Thiadiazole-2,5-diyl)bis(sulfanediyl)]bis (methylene)}bis (2H-benzo[h] chromen-2-one) (**9b**)*

Brownish yellow solid, yield (1.8 gm, 65%), mp 198–200 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 4.90 (s, 4H), 6.56 (s, 2H), 7.65–7.76 (m, 3H), 7.83–7.93 (m, 3H), 7.94–8.10 (m, 4H), 8.20–8.50 (br s, 2H);ESI-MS: *m/z* calculated for $C_{30}H_{18}N_2O_4S_3$: 566, found: 567 (M^++1); Anal. Calcd. For $C_{30}H_{18}N_2O_4S_3$: C, 63.59; H, 3.20; N, 4.94. Found: C, 63.34; H, 3.39; N, 5.03.

*4.1.31. Synthesis of 4-hydroxy-2-sulfanyl-6-phenylpyrimidine-5-carbonitrile (**10**)*

Compound **10** was synthesized according to the published method (Shaquiquzzaman et al., 2012).

*4.1.32. Synthesis of 4-hydroxy-2-{[(6-methyl-2-oxo-2H-chromen-4-yl)methyl]sulfanyl}-6-phenylpyrimidine-5-carbonitrile (**11**)*

Triethylamine (1 ml, 0.01 mol) was added to a solution of **10** (2.29 g, 0.01 mol) in dry tetrahydrofuran. The mixture was stirred at room temperature for 1 h then solution of **3a** (2.52 g, 0.01 mol) in dry tetrahydrofuran was added portionwise and the reaction mixture was stirred for additional 24 h at room temperature then the solvent was concentrated under reduced pressure and the residue was poured onto crushed ice with stirring. The precipitate was filtered off and recrystallized from ethanol to give compound **11**.

Brownish white solid (2.44 gm, 61%), mp 240–242 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.33 (s, 3H), 4.59 (s, 2H), 6.57 (s, 1H), 7.32 (s, 1H), 7.45 (br s, 4H), 7.79 (br s, 3H), 10.05 (s, 1H, D_2O-exchangable);ESI-MS: *m/z* calculated for $C_{22}H_{16}N_3O_3S$: 401.1, found: 402.97 (M^++1); Anal. Calcd. For $C_{22}H_{16}N_3O_3S$: C, 65.82; H, 3.77; N, 10.47. Found: C, 65.69; H, 3.67; N, 10.73.

*4.1.33. Synthesis of 4-chloro-2-{[(6-methyl-2-oxo-2H-chromen-4-yl) methyl]sulfanyl}-6-phenylpyrimidine-5-carbonitrile (**12**)*

Phosphorous oxychloride (10 ml) was added dropwise with stirring to a flask charged with compound **11** (4.4 g, 0.011 mol) at 0 °C. The mixture was stirred at room temperature for 0.5 h then refluxed on water bath at 70 °C for 6 h. The reaction mixture was cooled, and poured onto crushed ice. The formed solid was filtered and recrystallized from methylene chloride to give compound **12**.

Light brown solid, (3 gm, 65%), mp 231–233 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.37 (s, 3H), 4.76 (s, 2H), 6.62 (s, 1H), 7.34 (br s, 1H), 7.48–7.56 (m, 3H), 7.65 (br s, 1H), 7.83–7.90 (m, 3H);ESI-MS: *m/z* calculated for $C_{22}H_{15}ClN_3O_2S$: 419, found: 420.96 (M^++1); Anal. Calcd. For $C_{22}H_{15}ClN_3O_2S$: C, 62.93; H, 3.36; N, 10.01. Found: C, 63.12; H, 3.30; N, 10.15.

*4.1.34. Synthesis of 4-substituted-2-{[(6-methyl-2-oxo-2H-chromen-4-yl)methyl]sulfanyl}-6-phenylpyrimidine-5-carbonitrile (**13a** and **13b**)*

Solution of compound **12** (2 gm, 0.005 mol) in absolute ethanol was added portionwise to a well stirred solution of the appropriate amine (0.005 mol) and triethyl amine (0.5 ml, 0.005 mol) in absolute ethanol (10 ml). The mixture was refluxed for 5 h then the solvent was removed under reduced pressure and the remained solid was washed with cold water and purified by recrystallization from ethanol.

Compound **13a** was prepared by stirring the reaction mixture for 8 h at room temperature and no need to reflux, then left to stand overnight at room temperature. The formed precipitate was filtered, air dried and recrystallized from ethanol.

*4.1.35. 4-Hydrazinyl-2-{[(6-methyl-2-oxo-2H-chromen-4-yl) methyl]sulfanyl} -6-phenylpyrimidine-5-carbonitrile (**13a**)*

Light brown solid, (1.49 gm, 72%), mp 207–209 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.34 (s, 3H), 4.68 (s, 2H), 5.10 (s, 2H, D_2O exchangeable) 6.65 (s, 1H), 7.32 (br s, 1H), 7.48–7.53 (m, 4H), 7.76 (br s, 3H), 9.80(s, 1H, D_2O-exchangeable);ESI-MS: *m/z* calculated for $C_{22}H_{18}N_5O_2S$: 415.1, found: 416.95 (M^++1); Anal. Calcd. For $C_{22}H_{18}N_5O_2S$: C, 63.60; H, 4.12; N, 16.86. Found: C, 63.79; H, 4.03; N, 16.80.

*4.1.36. 2-{[(6-Methyl-2-oxo-2H-chromen-4-yl)methyl]sulfanyl}-4-Phenylamino-6-phenylpyrimidine-5-carbonitrile (**13b**)*

Brownish yellow solid, (1.7 gm, 72%), mp 287–289 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ:2.35 (s, 3H), 4.50 (s, 2H), 6.24 (s, 1H), 7.04–7.31 (m, 4H), 7.45–7.56 (m, 7H), 7.85 (br s, 2H) 9.95(s, 1H, D_2O-exchangeable); ESI-MS: *m/z* calculated for $C_{28}H_{21}N_4O_2S$: 476.1, found: 477 (M^++1); Anal. Calcd. For $C_{28}H_{21}N_4O_2S$: C, 70.57; H, 4.23; N, 11.76. Found: C, 70.43; H, 4.15; N, 11.84.

*4.1.37. Synthesis of 4-substituted-2-{[(6-methyl-2-oxo-2H-chromen-4-yl) methyl]sulfanyl}-6-phenylpyrimidine-5-carbonitrile (**13c** and **13d**)*

A mixture of morpholine (0.8 gm, 0.01 mol) or piperazine (5.16 gm, 0.06 mol) and anhydrous potassium carbonate (1.38 gm, 0.01 mol) in absolute ethanol (8 ml) was stirred for 0.5 h. A solution of compound **12** (4.19 gm, 0.01 mol) in absolute ethanol (12 ml) was added portionwise. The reaction mixture was refluxed for 8 h, allowed to cool then filtered. The filtrate was concentrated under reduced pressure then poured onto crushed ice. The formed precipitate was filtered, washed with water and recrystallized from water/acetone.

Compound **13d** was prepared using the same procedure but the filtrate was stirred with 2N hydrochloric acid before filtration,

*4.1.38. 2-{[(6-Methyl-2-oxo-2H-chromen-4-yl)methyl]sulfanyl}-4-(morpholin -4-yl)-6-phenylpyrimidine-5-carbonitrile (**13c**)*

Yellowish orange solid, (2.8 gm, 60%), m p 220–222 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.37 (s, 3H), 3.60 (br s, 4H), 3.84 (br s, 4H) 4.66 (s, 2H), 6.57 (s, 1H), 7.36 (br s, 2H), 7.40–7.65 (m, 3H), 7.80(br s, 3H); ESI-MS: *m/z* calculated for $C_{26}H_{23}N_4O_3S$: 470.1, found: 471.97 (M^++1); Anal. Calcd. For $C_{26}H_{23}N_4O_3S$: C, 66.37; H, 4.71; N, 11.91. Found: C, 66.44; H, 4.58; N, 11.98.

*4.1.39. 2-{[(6-Methyl-2-oxo-2H-chromen-4-yl)methyl]sulfanyl}-4-phenyl-6-(piprazinyl-1-yl)pyrimidine-5-carbonitrile (**13d**)*

Light orange solid, (2.5 gm, 53%), mp 215–217 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.37 (s, 3H), 2.82 (br s, 4H), 3.82 (br s, 4H), 4.65 (s, 2H), 6.57 (s, 1H), 7.35 (m, 1H), 7.47–7.58 (m, 4H), 7.73 (br s, 1H, D_2O exchangeable), 7.81 (br s, 3H); ESI-MS: *m/z* calculated for $C_{26}H_{24}N_5O_2S$: 469.2, found: 470 (M^++1); Anal. Calcd. For $C_{26}H_{24}N_5O_2S$: C, 66.50; H, 4.94; N, 14.91. Found: C, 66.41; H, 4.87; N, 15.11.

4.2. Biology

All final compounds were evaluated for their antitumor activity against human breast cancer cell line (MCF-7) and hepatocellular carcinoma cell line (HepG-2) adopting MTT assay [1]. All materials were obtained from Sigma Chemical Co (USA). The cell lines were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. 5-Fluorouracil was used as a standard anticancer drug for comparison. MTT assay is a colorimetric assay based on the reduction of the 3-(4,5-dimethyl-2-thia zolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Cell lines were cultured in RPMI-1640 medium with

10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. The cell lines were seeded in a 96-well plate at a density of 1×10^4 cells/well at 37 °C for 48 h under 5% CO_2. After incubation the cells were treated with different concentration of the tested compounds and incubated for 24 h, then 20 μl of MTT solution at 5 mg/ml was added and incubated for 4 h. 100 μl of dimethyl sulfoxide (DMSO) is added into each well to dissolve the purple formazan formed. The absorbance of each well was measured by a microplate reader (EXL 800 USA) using a test wavelength of 570 nm. The results was expressed as IC_{50}, which is the concentration of the drugs inducing a 50% inhibition of cell growth of treated cells when compared to the growth of control cells. The IC_{50} values were calculated using different concentrations of the tested compounds (Mosmann, 1983).

4.3. Molecular docking

The docking studies and modeling calculations were done using 'MOE version 2009.10 release of Chemical Computing Group's' which was operated under Windows XP operating system installed on an Intel Pentium IV PC with a 2.8 MHz processor and 512 RAM. The tested compounds were built in 2D using ChemBiooffice suite and geometric optimization was done using Hyperchem, then subjected to docking simulation. The X-ray crystallographic structure of CDK2 protein enzyme was obtained from the Protein Data Bank; code '1KE9.pdb'.

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