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Synthesis and biological evaluation of novel fused triazolo[4,3-a] pyrimidinones

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Abstract: The reaction of thione **3** or its 2-methylthio derivative **4** with hydrazonoyl halides **5a**–**l**, in the presence of triethylamine, yielded the corresponding triazolo[4,3-a]pyrimidin-5(1H)-ones **8a**–**l**. The structure of compounds **8a**–**l** was further confirmed by the reaction of **3** with the appropriate active chloromethylenes **11a**–**c** followed by coupling of the products with benzenediazonium chloride to afford the azo-coupling products **6b**, **f**, and **j**, which were converted in situ to **8b**, **f**, and **j**. 2-Hydrazinyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(3H)-one (**13**) was prepared and condensed with different aldehydes **14a**–**f** to give the corresponding hydrazone derivatives **15a**–**f**. Oxidative cyclization of the hydrazones **15a**–**f** give the corresponding triazolo[4,3-a] pyrimidin-5(1H)-one derivatives **16a**–**f**.

The antimicrobial activity of the products was evaluated and the results revealed that compounds **8f** and **15f** showed strong activity against gram-positive bacteria while compound **15d** showed the highest activity against gram-negative bacteria. Moreover, compounds **15b**, **8d**, **8e**, **8c**, **8l**, and **8j** exhibited significant antifungal activity. In addition, the antitumoral activity of the synthesized products against different cancer cell lines was determined and the results revealed that compound **12c** was the most active against MCF-7, HepG-2, HCT-116, and HeLa with IC_{50} values of 0.51, 0.72, 0.95, and 0.95, respectively, as compared with doxorubicin as positive control.

Key words: Triazolopyrimidinones, cyclizations, hydrazonyl chlorides, antimicrobial, anticancer activity

1. Introduction

It is known that cancer is one of the most dangerous diseases, caused by uncontrolled growth and spread of abnormal cells, initiated by viruses, smoking, chemicals, or diet.[1] Cancer can lead to death if left untreated. Therefore, many of the research efforts aim to develop new anticancer drugs.[2–6]

The 1,2,4-triazolopyrimidines have attracted growing interest due to their important pharmacological activities, such as antitumor potency,[7–12] antimalarial,[13] antimicrobial,[14–17] anti-inflammatory,[18] inhibition of kinase insert domain containing receptor (KDR kinase),[19] antifungal,[20] and macrophage activation.[21] In addition, triazolo[4,3-a]pyrimidine derivatives were reported to be useful as antihypertensive,[22] anxiolytic,[23] and cardiovascular[24,25] agents.

In view of these reports and in continuation of our previous work on the synthesis of bioactive heterocyclic compounds,[26–32] we were interested in the synthesis of new fused triazolopyridinones to investigate their antimicrobial and cytotoxic potential activities.

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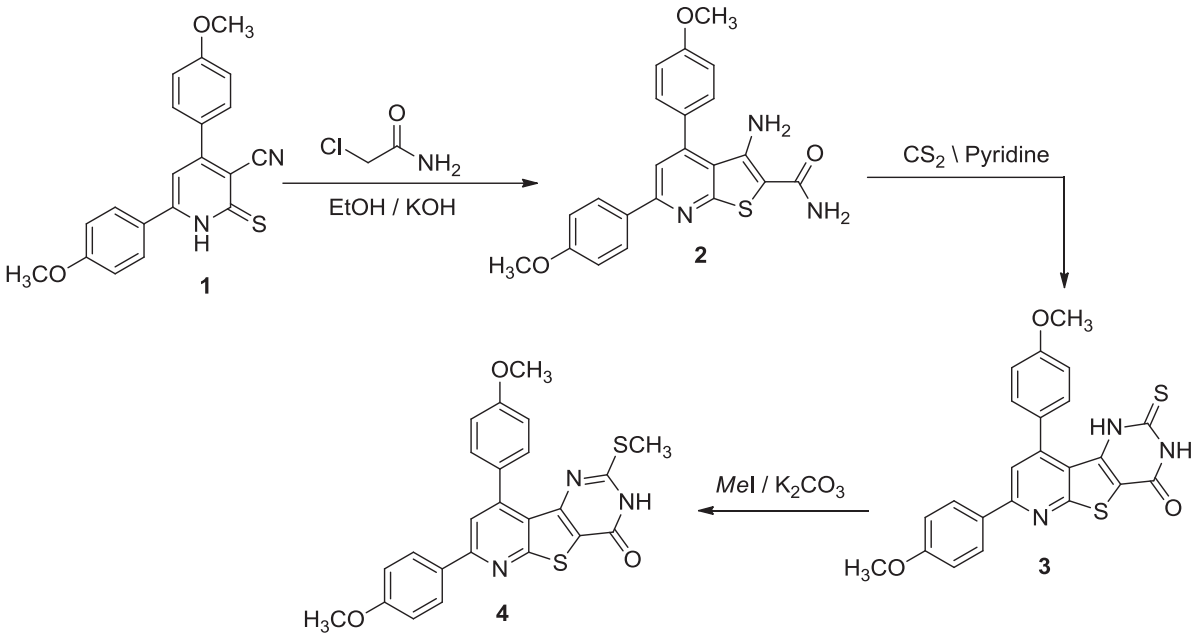
2. Results and discussion

2.1. Chemistry

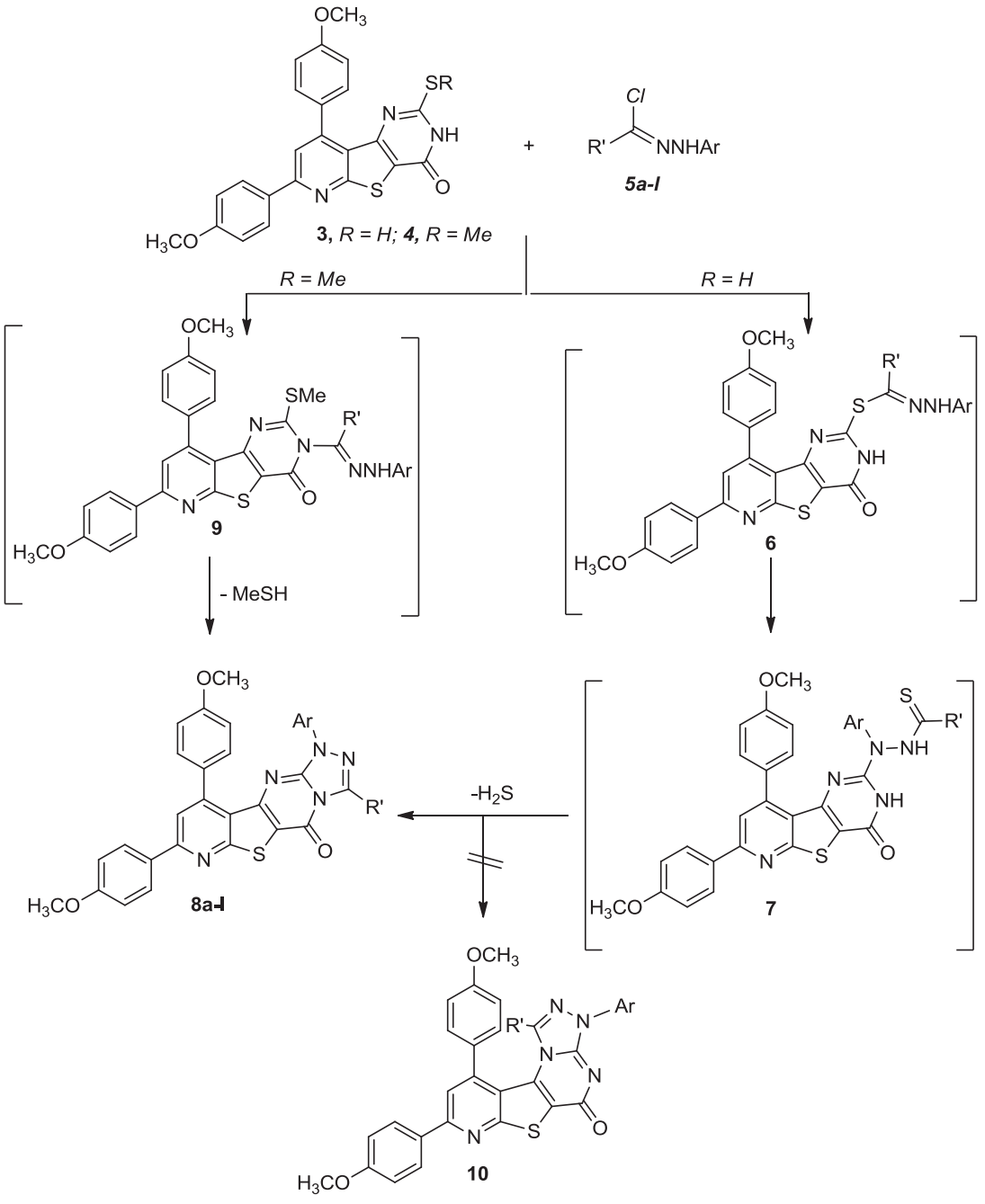
The starting compound 7,9-bis(4-methoxyphenyl)-2-thioxo-2,3-dihydropyrido[3',2':4,5] thieno[3,2-*d*]pyrimidin-4(1*H*)-one (**3**) was prepared by adopting a reported procedure[33] as depicted in Scheme 1. Thus, the reaction of 4,6-di(4-methoxyphenyl)-2-thioxo-1,2-dihydropyridine-3-carbonitrile (**1**)[34] with chloroacetamide in refluxed ethanol containing KOH afforded compound **2**. The structure of compound **2** was confirmed on the basis of its spectral data (see Experimental). Treatment of the latter compound with CS_2 in pyridine followed by acidification led to the formation of the starting material **3**. The structure of **3** was evidenced by its spectral data (mass, IR, ^{1}H NMR) and thermal analysis. Its IR spectrum revealed the absorption bands of two –NH and CO in the regions 3459, 3359, and 1691 cm^{-1}, respectively while its ^{1}H NMR spectrum showed two characteristic signals at $\delta =$ 9.29 and 12.99 ppm (D_2O exchangeable) assignable to two –NH protons.



Scheme 1. Synthesis of thione **3** and its methylthio derivative **4**.

The methylthio derivative **4** was prepared from the reaction of thione **3** with methyl iodide in the presence of anhydrous K_2CO_3. The ^{1}H NMR spectrum of compound **4** showed the signals of S–CH_3 and NH at $\delta =$ 3.56 and 12.96 ppm, respectively.

Reaction of **3** with each of **5a**–**l** was carried out in dioxane, in the presence of triethylamine, under reflux until hydrogen sulfide ceased to evolve, affording, in each case, one isolable product as evidenced by TLC analysis (Scheme 2). The isolated products were assigned the structure of pyridothieno[3,2-*d*]triazolo-pyrimidin-5(1*H*)-ones **8a**–**l** rather than its isomeric structure pyridothieno[2,3-*e*]triazolo-pyrimidin-5(3*H*)-one **10** based on their elemental analyses and IR, ^{1}H NMR, and ^{13}C NMR spectra (see Experimental). For example, the δ values (δ = 164 ppm) for the carbonyl carbon signal in the ^{13}C NMR spectra of **8a** are similar to those of compounds of type **A** (δ = 161–164 ppm) and different from those of their isomers having structure **B** (δ = 170–175 ppm)[35] (Figure 1). This finding excludes structure **10** for the products.



R' / Z (Ar = ZC_6H_4)

a, Ph / H; b,CO*Me* / H; c,CO*Me* / 4-CH_3 ; *d,*CO*Me* / 4-Cl; e, CO*Me* / 4-NO_2;
f, COO*Et* / H; g,COO*Et* / 4-CH_3; *h,* COO*Et* / 4-Cl; i,COO*Et* / 4-OCH_3;
*j,*CONH*Ph* / H; k,CONH*Ph* / 4-CH_3; l, CONH*Ph* / 4-Cl

Scheme 2. Synthesis of pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidin-5(1 H)-ones (**8a**–**l**).

Compound **8** was further evidenced by alternate synthesis, via refluxing 2-methylthio derivative **4** with **5a**–**l** in the presence of triethylamine until the evolution of methanethiol ceased and products formed that

proved identical in all respects (IR, MS, mp, and mixed mp) with **8a**–**l**. The assumption that the latter reaction proceeds through the amidrazone intermediate **9** is compatible with the literature.[36]

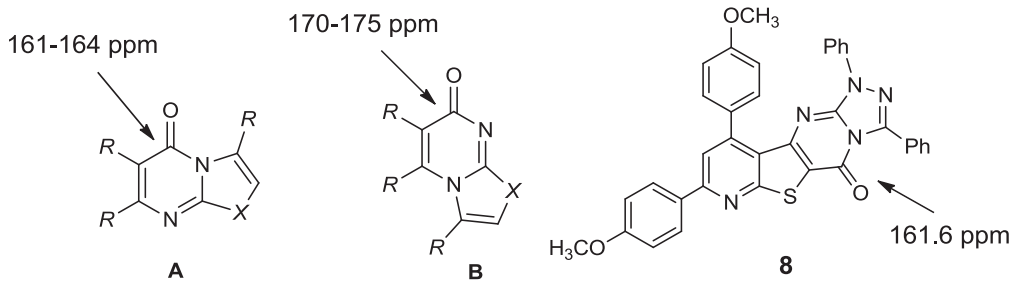


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Figure 1. ^{13}C NMR shifts of strategic carbon atoms.

Formation of compounds **8** could be started with the formation of thiohydrazonate esters **6** from hydrazonoylation of thione **3**, followed by Smiles rearrangement[37] to form the respective thiohydrazides **7**. Cyclization of **7** followed by removal of H_2S yielded **8** (Scheme 2). Alternatively, the formation of **8** from methylthio derivative **4** and hydrazonoyl halides **5** can be accomplished via cyclization of the amidrazone **9** (Scheme 2).

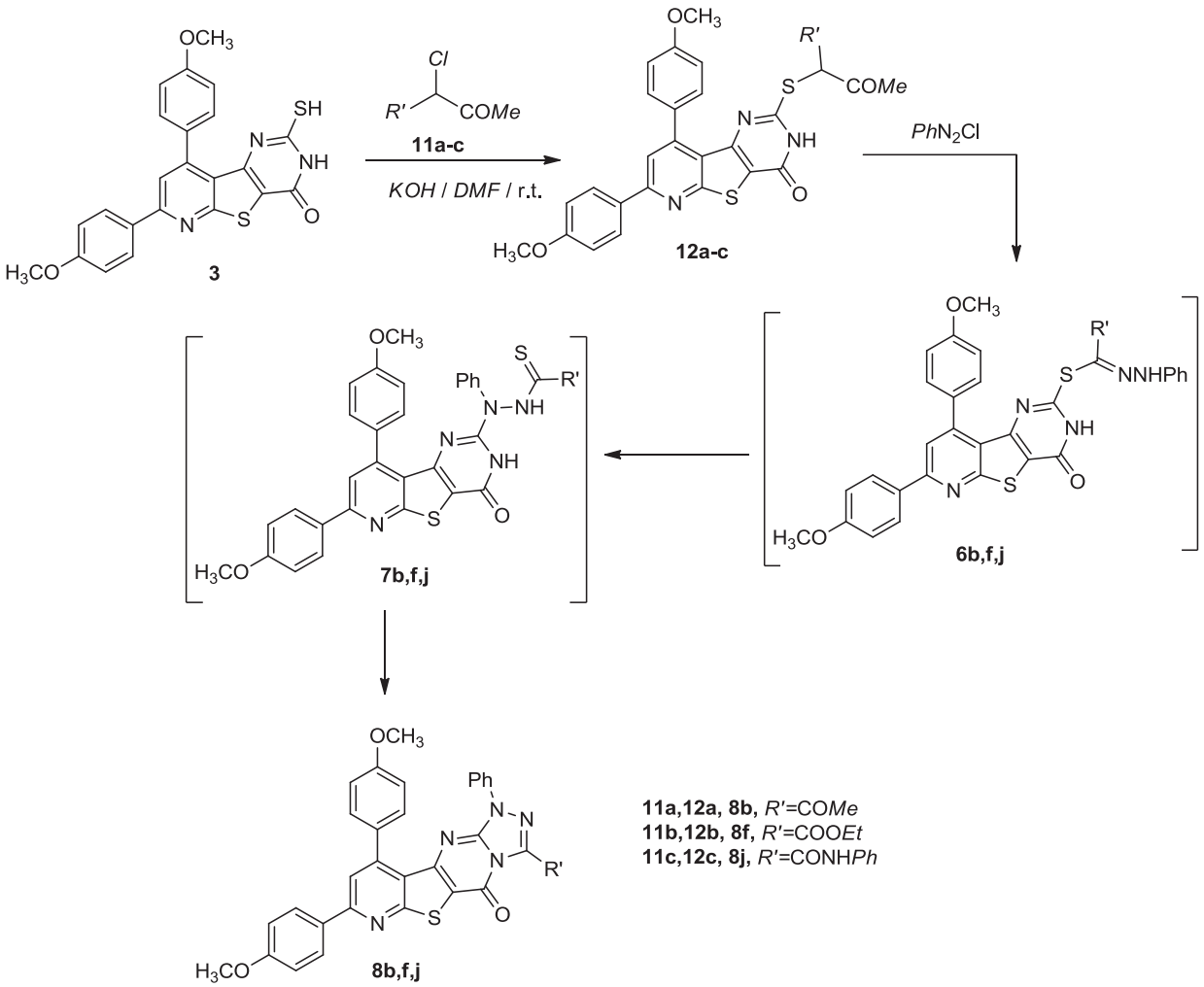
The involvement of **6** and **7** as intermediates in the formation of **8** was evidenced by alternate synthesis of **8b**, **f**, and **j** (Scheme 2). Thus, treatment of **3** with each of the active chloromethylene compounds **11a**–**c** in KOH/DMF at room temperature yielded the substitution products **12a**–**c** (Scheme 3). The structure of the latter products was elucidated by its microanalysis and spectral data (mass, IR, ^{1}H NMR). The ^{1}H NMR data showed singlet signals at δ = 2.34 and 4.01 ppm assignable to the methyl and methine protons, respectively, in addition to the characteristic signals corresponding to COMe, COOEt, and CONHPh groups in the compounds **12a**–**c**, respectively. The formation of **12a**–**c** from the reaction of **3** with **11a**–**c** (Scheme 3) is analogous to S-alkylation reactions reported for 2-thioxopyrimidines.[38]

Coupling of **12a**–**c** with benzenediazonium chloride yielded the corresponding thiohydrazonate esters **6b**, **f**, and **j**, which undergo in situ Smiles rearrangement to give the intermediates **7b**, **f**, and **j**, which could be cyclized into the corresponding **8b**, **f**, and **j**. This finding indicates that **6** and **7** are intermediates in the studied reactions of **3** with **5a**–**l**.

Finally, the suggestion that the site of cyclization of the thiohydrazide intermediates **7** involves N-3 to give **8** is consistent with literature reports.[39–41]

2-Hydrazinyl-7,9-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(3*H*)-one (**13**) was prepared by refluxing compound **4** with $NH_2NH_2.H_2O$ in DMF. Condensation of compound **13** with different aldehydes **14a**–**f** in acetic acid gave the corresponding hydrazone derivatives **15a**–**f** (Scheme 4). The mass spectra of the isolated products **15a**–**f** showed the molecular ion peaks at the expected m/z values. Their IR spectra showed the disappearance of the NH_2 group, and revealed in each case a carbonyl band in the region 1651–1670 cm^{-1} and two bands at 3444–3448 and 3348–3363 cm^{-1} assignable to two –NH groups. Furthermore, the ^{1}H NMR spectra showed, in each case, the presence of the azomethine and two –NH protons at δ = 8.11–8.20, 10.98–11.16, and 11.69–11.89 ppm, respectively.

Oxidative cyclization of the hydrazone derivatives **15a**–**f** with bromine in acetic acid in the presence of sodium acetate at room temperature yielded in each case one isolable product **16a**–**f** (Scheme 4). The products



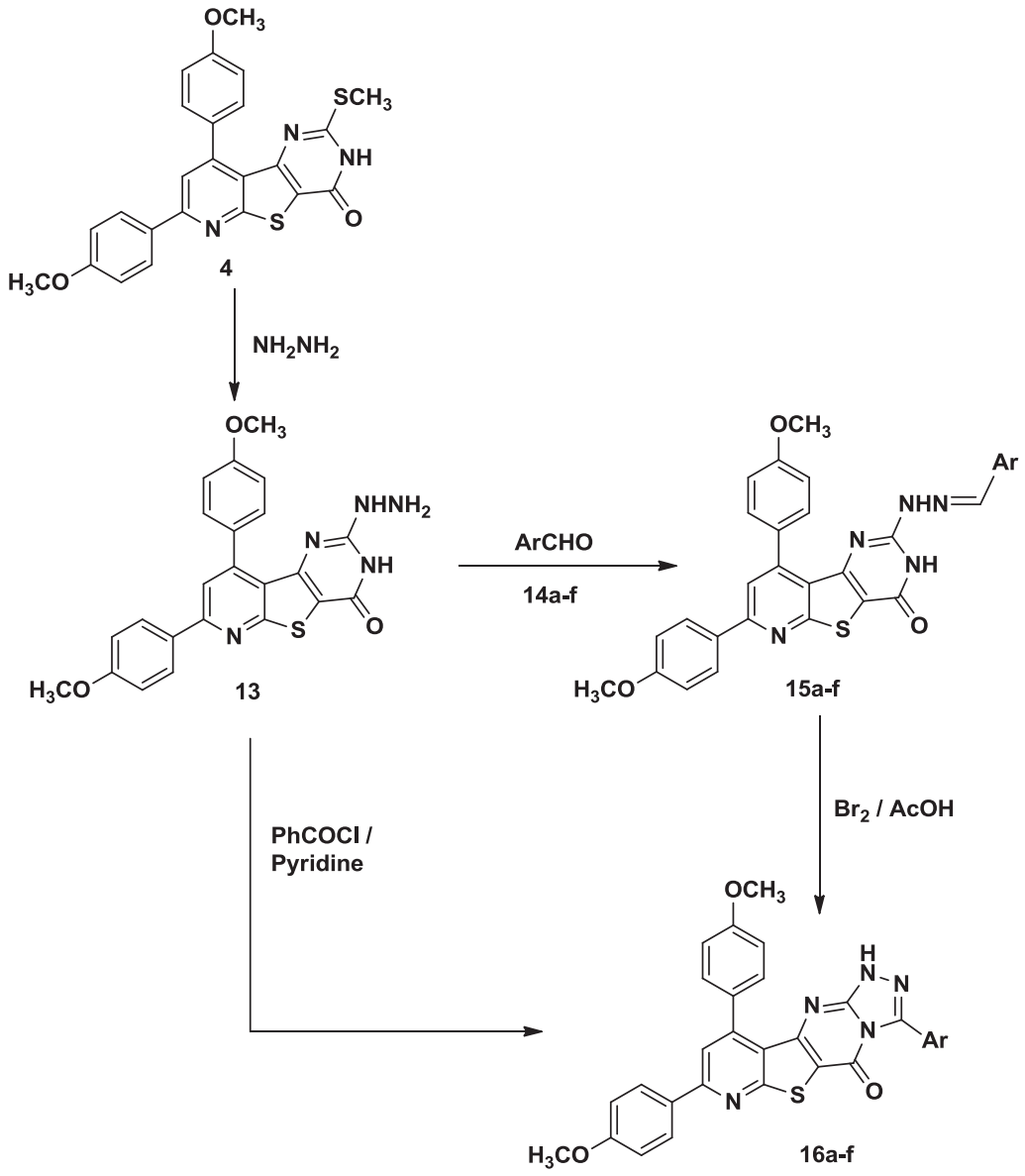
Scheme 3. Alternative synthesis of pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidin-5(1*H*)-ones (**8b**, **f**, and **j**).

16a–**f** were deduced from their spectral data (IR, ^{1}H NMR, and ESI-MS) and elemental analyses, which are listed in the Experimental part. In addition, compound **16a** was synthesized by an alternative method via reaction of compound **13** with benzoyl chloride in pyridine.

3. Biological activity

3.1. Antimicrobial evaluation

The newly synthesized target compounds were evaluated for their in vitro antibacterial activity against two gram-positive bacterial species (*Bacillus subtilis* and *Staphylococcus aureus*), two gram-negative bacterial species (*Escherichia coli* and *Pseudomonas aeruginosa*), two moulds (*Aspergillus fumigatus* and *Syncephalastrum racemosum*), and two yeasts (*Candida albicans* and *Geotrichum candidum*) using a modified well diffusion method. The organisms were tested against the activity of solutions of concentrations (5 mg/mL) and using inhibition zone diameter in millimeters as criterion for the antimicrobial activity (agar well diffusion method). The results of testing for antibacterial and antifungal effects are summarized in Tables 1 and 2. As shown by these results, the new fused triazolopyrimidinone derivatives tested displayed variable in vitro antibacterial



Scheme 4. Synthesis of pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidin-5(1*H*)-one (**16a**–**f**).

and antifungal actions. In general, the chemical structure of the whole molecule, comprising the nature of the heterocyclic system as well as the type of the substituted function present in the heterocyclic ring structure, has a pronounced effect on antimicrobial activity. Most of the corresponding substituted analogues produced higher inhibitory effects against bacteria similar or superior to the reference drug tetracycline.

From the screening results, it can be seen that compounds **8f** and **15f** showed the highest activity against gram-positive bacteria and compound **15d** showed the highest activity against gram-negative bacteria. The rest of the compounds showed good to moderate activity against the tested bacteria compared with the standard drugs.

Table 1. In vitro antibacterial activity of the tested compounds by well diffusion agar assay expressed as inhibition zone diameter (mm) in the form of mean ± SD.

Tested compounds	Gram-positive bacteria		Gram-negative bacteria	
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*
3	17.9 ± 0.5	22.8 ± 0.8	19.3 ± 0.7	13.2 ± 0.4
8a	18.2 ± 0.7	22.4 ± 0.4	18.1 ± 0.9	11.6 ± 0.9
8b	11.9 ± 0.3	17.2 ± 0.6	10.6 ± 0.3	8.1 ± 0.6
8c	19.7 ± 0.8	24.7 ± 0.7	18.2 ± 0.7	13.4 ± 0.7
8d	18.5 ± 0.9	25.6 ± 1.3	14.7 ± 0.6	10.3 ± 0.5
8e	21.7 ± 0.8	24.1 ± 0.9	22.3 ± 1.1	17.2 ± 0.9
8f	**30.8** ± 1.2	26.8 ± 0.7	24.6 ± 0.9	18.4 ± 0.8
8g	19.2 ± 0.4	25.6 ± 0.8	20.1 ± 0.8	13.3 ± 0.7
8h	18.3 ± 0.6	23.9 ± 0.6	10.7 ± 0.4	8.5 ± 0.4
8i	13.4 ± 0.2	9.7 ± 0.3	21.5 ± 0.9	13.7 ± 0.2
8j	19.1 ± 0.5	12.8 ± 0.2	22.3 ± 1.1	16.6 ± 0.9
8k	18.7 ± 0.8	17.2 ± 0.7	20.4 ± 0.8	12.8 ± 0.6
8l	**23.6** ± 0.7	**25.9** ± 1.3	23.8 ± 0.7	12.3 ± 0.4
12a	12.1 ± 0.8	9.7 ± 0.4	7.2 ± 0.3	7.4 ± 0.3
12b	21.7 ± 0.6	17.9 ± 0.8	15.8 ± 0.4	14.1 ± 0.8
12c	15.3 ± 0.5	21.8 ± 1.1	17.6 ± 0.6	11.4 ± 0.5
15a	21.8 ± 0.9	25.6 ± 0.8	24.1 ± 0.9	16.9 ± 0.7
15b	20.2 ± 0.7	18.9 ± 0.5	10.8 ± 0.5	14.7 ± 0.3
15c	14.2 ± 0.4	19.7 ± 0.9	10.9 ± 0.4	7.6 ± 0.4
15d	21.3 ± 0.7	25.3 ± 1.1	**30.7** ± 1.3	24.6 ± 0.9
15e	13.1 ± 0.5	19.8 ± 0.8	10.2 ± 0.6	11.5 ± 0.4
15f	**29.3** ± 1.1	24.3 ± 0.7	19.7 ± 0.8	13.6 ± 0.6
Tetracycline	28.7 ± 0.5	26.4 ± 0.7	30.2 ± 0.6	27.4 ± 0.8

Interestingly, compound **15b** showed higher inhibitory activity against *Aspergillus fumigates* compared with amphotericin B reference drug. Despite promising in vitro antifungal activity of some of the newly synthesized compounds, only compounds **8d**, **8e**, **8c**, **8l**, and **8j** among the compounds tested exhibited high antifungal activity as compared with those of the reference drug against yeast species. Compound **8c** also exhibited a higher inhibitory effect than the reference drug, amphotericin B.

The mean values of the inhibition zone diameter obtained for these compounds suggest that all synthesized compounds possess significant antimicrobial activity against most test organisms used in these assays (Tables 1 and 2); therefore, minimum inhibitory concentration (MIC) of various synthesized compounds was evaluated in vitro using the two-fold serial dilution technique, while the lowest concentration showed no growth as the MIC. The fungicides amphotericin B and gentamicin as well as the bactericides ampicillin and tetracycline were used as references to evaluate the potency of the tested compounds under the same conditions. The results of MIC are reported in Table 3.

Table 2. In vitro antifungal activity of the tested compounds by well diffusion agar assay expressed as inhibition zone diameter (mm) in the form of mean ± SD.

Tested compounds	Filamentous fungi		Yeasts	
	Aspergillus fumigatus	*Syncephalastrum racemosum*	*Candida albicans*	*Geotrichum candidum*
3	11.4 ± 0.5	8.4 ± 0.9	-	-
8a	12.1 ± 0.7	10.3 ± 0.5	10.4 ± 0.9	8.5 ± 0.3
8b	-	-	-	-
8c	**26.5** ± 0.8	**24.2** ± 0.9	**26.4** ± 1.3	20.9 ± 0.8
8d	**29.3** ± 1.2	18.9 ± 0.5	**25.2** ± 0.8	**24.3** ± 0.9
8e	23.1 ± 0.9	19.2 ± 0.8	**24.5** ± 0.5	22.6 ± 1.1
8f	11.7 ± 0.8	8.1 ± 0.4	14.7 ± 0.9	13.2 ± 0.7
8g	26.2 ± 2.1	21.8 ± 0.9	18.3 ± 0.6	14.5 ± 0.3
8h	24.6 ± 0.7	18.3 ± 0.6	17.7 ± 1.1	14.1 ± 0.7
8i	-	-	11.5 ± 0.4	9.2 ± 0.5
8j	18.9 ± 0.6	12.7 ± 0.4	22.3 ± 0.9	16.3 ± 0.8
8k	26.5 ± 1.1	20.3 ± 0.8	21.7 ± 0.8	17.6 ± 0.9
8l	**28.3** ± 0.8	18.2 ± 0.5	23.2 ± 1.2	20.2 ± 0.7
12a	-	-	-	-
12b	8.9 ± 0.5	9.3 ± 0.7	-	-
12c	11.3 ± 0.7	8.8 ± 0.5	9.7 ± 0.4	8.4 ± 0.5
15a	8.9 ± 0.4	7.1 ± 0.9	-	-
15b	**29.8** ± 1.2	**22.3** ± 0.6	**20.6** ± 1.3	18.5 ± 1.1
15c	12.2 ± 0.6	8.7 ± 0.5	9.2 ± 0.6	7.6 ± 0.8
15d	18.2 ± 0.7	14.5 ± 0.4	11.3 ± 0.7	10.1 ± 0.9
15e	-	-	-	-
15f	11.4 ± 0.5	9.3 ± 0.3	9.8 ± 0.8	7.6 ± 0.3
Amphotericin B	27.1 ± 0.6	23.2 ± 0.9	17.9 ± 0.3	19 ± 0.5

Compound **15d** showed an appreciable broad spectrum of action against both gram-positive and gram-negative bacteria with an antibacterial potency higher than that of their reference drug. Compound **15d** reached the highest potency with MIC 100 and 50 μg/mL against *S. aureus*, *E. coli*, and *P. aureginosa*, respectively. Significant MIC values were determined for compounds **8c** and **15b** against the tested fungi. Based on the biological evaluation, most of the compounds tested, in particular **8c**, **8f**, **8l**, **15b**, and **15f**, may be considered new antimicrobial agents.

3.2. Cytotoxic activity

The in vitro growth inhibitory activity of the synthesized compounds was investigated in comparison with 5-fluorouracil, doxorubicin, and imatinib[42] as standard drugs. The data generated were used to plot a dose response curve of which the concentration of test compounds required to kill 50% of the cell population (IC_{50}) was determined and the results revealed that all the tested compounds showed inhibitory activity to the tumor

Table 3. Antimicrobial activity minimum inhibitory concentration (MIC μg/mL) of synthesized compounds compared with standard drugs.

Tested compounds	Minimum inhibitory concentration (μg/mL)							
	Gram +ve bacteria		Gram –ve bacteria		Fungi			
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. fumigatus*	*S. racemosum*	*C. albicans*	*G. candidum*
3	500	500	500	1000	1000	1000	-	-
8a	100	100	250	500	500	1000	1000	1000
8b	500	250	500	500	-	-	-	-
8c	150	150	200	250	125	150	150	150
8d	200	125	250	500	100	250	150	150
8e	200	150	200	250	150	200	150	250
8f	100	150	150	200	500	1000	500	500
8g	150	125	200	250	125	250	500	500
8h	200	150	1000	1000	150	150	250	250
8i	250	500	200	500	-	-	1000	1000
8j	200	500	125	250	150	500	250	250
8k	200	200	250	500	125	150	200	250
8l	125	125	125	500	100	250	200	250
12a	500	500	1000	1000	-	-	-	-
12b	250	250	250	250	500	500	-	-
12c	250	150	150	250	250	500	500	500
15a	150	125	125	250	500	1000	-	-
15b	150	150	250	250	50	100	125	250
15c	250	250	250	500	250	500	500	500
15d	125	100	50	50	200	250	250	250
15e	500	250	500	500	-	-	-	-
15f	100	100	200	250	500	500	500	500
Tetracycline	50	50	50	50	-	-	-	-
Ampicillin	100	250	100	100	-	-	-	-
Griseofulvin	-	-	-	-	100	250	250	200
Amphotericin B	-	-	-	-	50	100	100	100

cell lines in a concentration dependent manner. Cytotoxic activity was expressed as the mean IC_{50} of six replicates in three independent experiments. The results are represented in Table 4 and Figures 2–4 show that compounds **12a**, **12b**, and **12c** have the highest cytotoxic activity against the two tumor cell lines MCF-7 and HepG-2, compared with reference drugs; thus these compounds were evaluated for their inhibitory effect on HCT-116 and HeLa cell lines. Moreover, compound **12c** was the most active against MCF-7, HepG-2, HCT-116, and HeLa with IC_{50} values of 0.51, 0.72, 0.95, and 0.95, respectively, as compared with doxorubicin. Interestingly, compounds **12a**, **12b**, **12c**, **15a**, and **15c** exhibited 48- to 1.07-fold more potent antitumor activity than imatinib against breast carcinoma (MCF-7) cell lines and were the most active among their analogues. Furthermore, compounds **15a**, **8f**, and **15c** showed cytotoxic effects against HepG2 comparable to imatinib.

Table 4. The in vitro inhibitory activity of tested compounds against tumor cell lines expressed as IC_{50} values (μg/mL) ± standard deviation from six replicates.

Tested compounds	Tumor cell lines			
	MCF-7	HepG2	HCT-116	HeLa
3	48.5	> 50	-	-
8a	44.9	41.0	-	-
8b	32.5	32.8	-	-
8c	37.9	37.9	-	-
8d	29.9	35.1	-	-
8e	36.4	38.4	-	-
8f	35.3	24.7	-	-
8g	39.7	40.2	-	-
8h	46.7	32.0	-	-
8i	36.4	41.1	-	-
8j	42.8	36.6	-	-
8k	26.1	35.7	-	-
8l	24.9	28.5	-	-
12a	3.5	5.0	9.3	11.6
12b	3.8	4.0	10.9	11.8
12c	0.51	0.72	0.95	0.95
15a	23.1	26.7	-	-
15b	41.6	44.9	-	-
15c	20.3	24.0	-	-
15d	37.8	37.8	-	-
15e	34.6	37.9	-	-
15f	24.7	27.9	-	-
Doxorubicin	0.46	0.42	0.46	0.63
Imatinib	24.6	18.9	9.7	34.1
5-Fluorouracil	3.9	4.6	4.3	6.8

Moreover, compounds **8a**, **8b**, **8c**, **8d**, **8e**, **8g**, **8h**, **8i**, **8j**, **8k**, **15b**, **15d**, and **15e** were less active than imatinib.

In light of the results presented in this work and taking into account that this preliminary study did not produce conclusive evidence regarding a structure antimicrobial activity relationship, we focused our attention on the most promising compounds, **8c**, **8f**, **8d**, and **15d**, as an interesting starting point for the development of a new class of antimicrobial agents. However, compounds **12c**, **12a**, and **12b** exhibited promising inhibitory activity against the four tested tumor cells. We think that research in this direction should be encouraged in order to broaden the applicability of these new heterocyclic frameworks to serve as leads for designing novel chemotherapeutic agents.

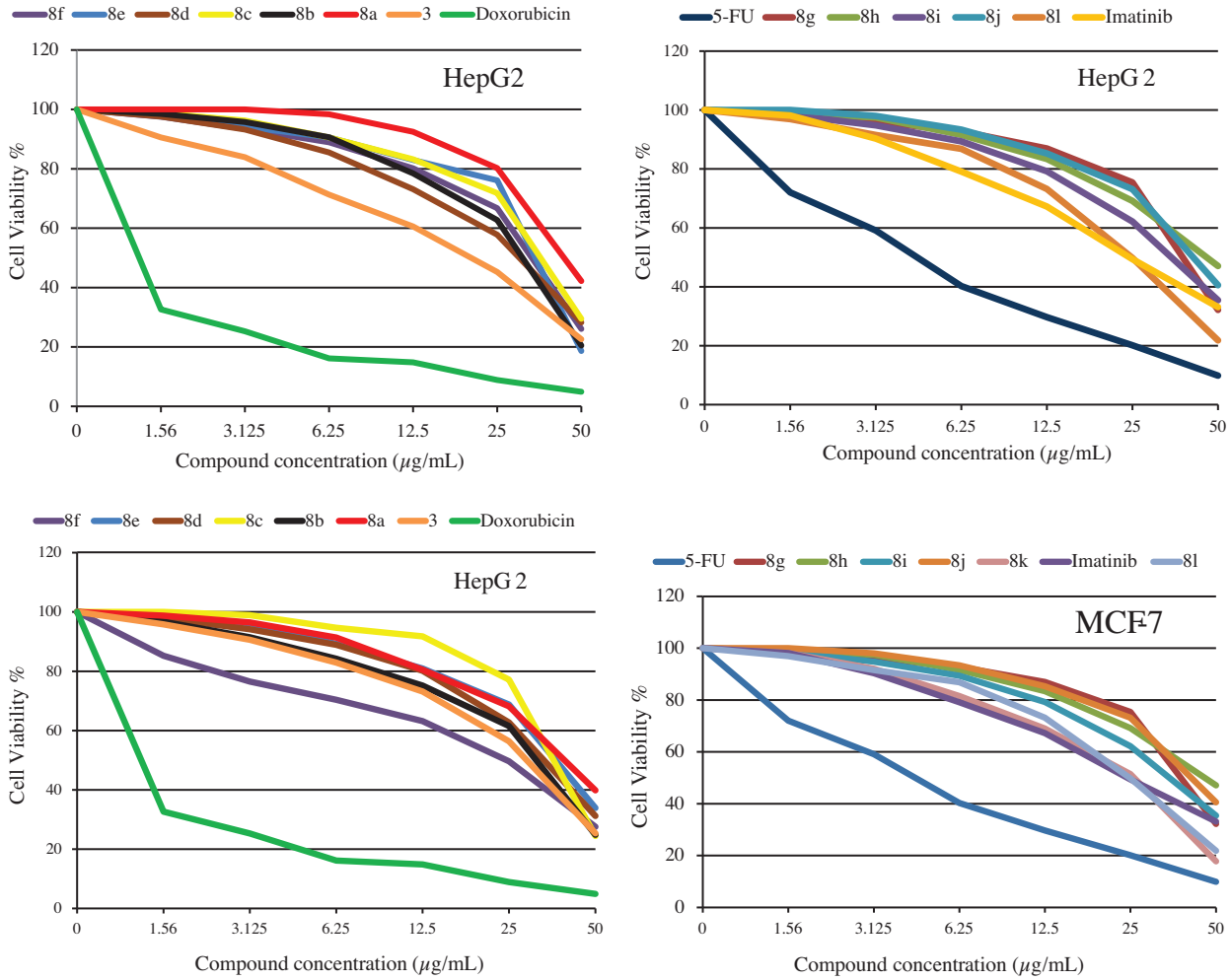


Figure 2. The dose response curve showing the in vitro inhibitory activity of compounds of **8** series (**8a**–**8l**) against (A and B) hepatocellular carcinoma (HepG2) and (C and D) breast carcinoma (MCF-7) cell lines.

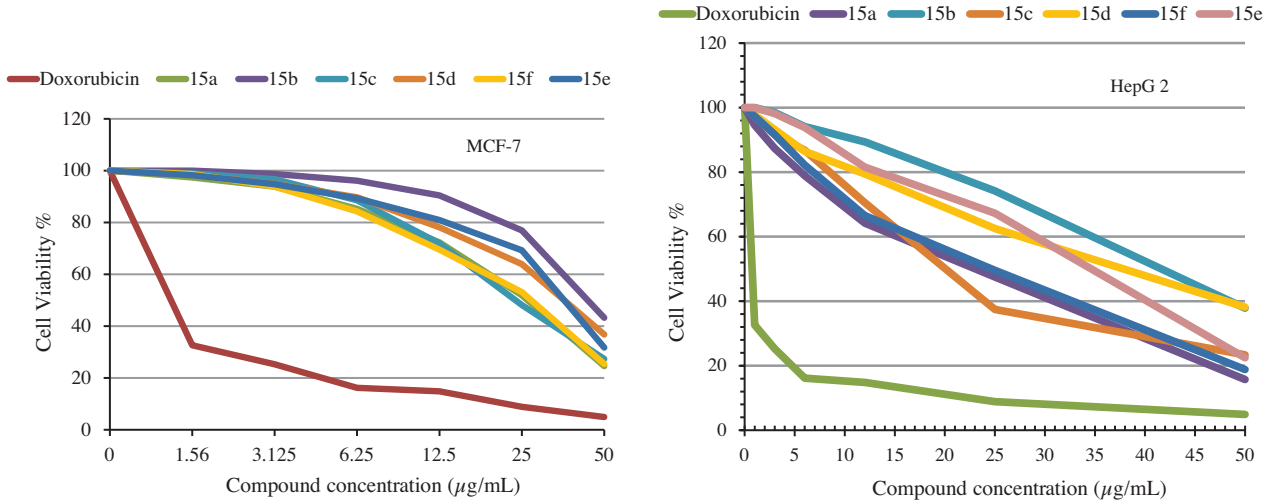


Figure 3. The dose response curve showing the in vitro inhibitory activity of compounds **15a**–**15f** against (A) breast carcinoma (MCF-7) and (B) hepatocellular carcinoma cell lines.

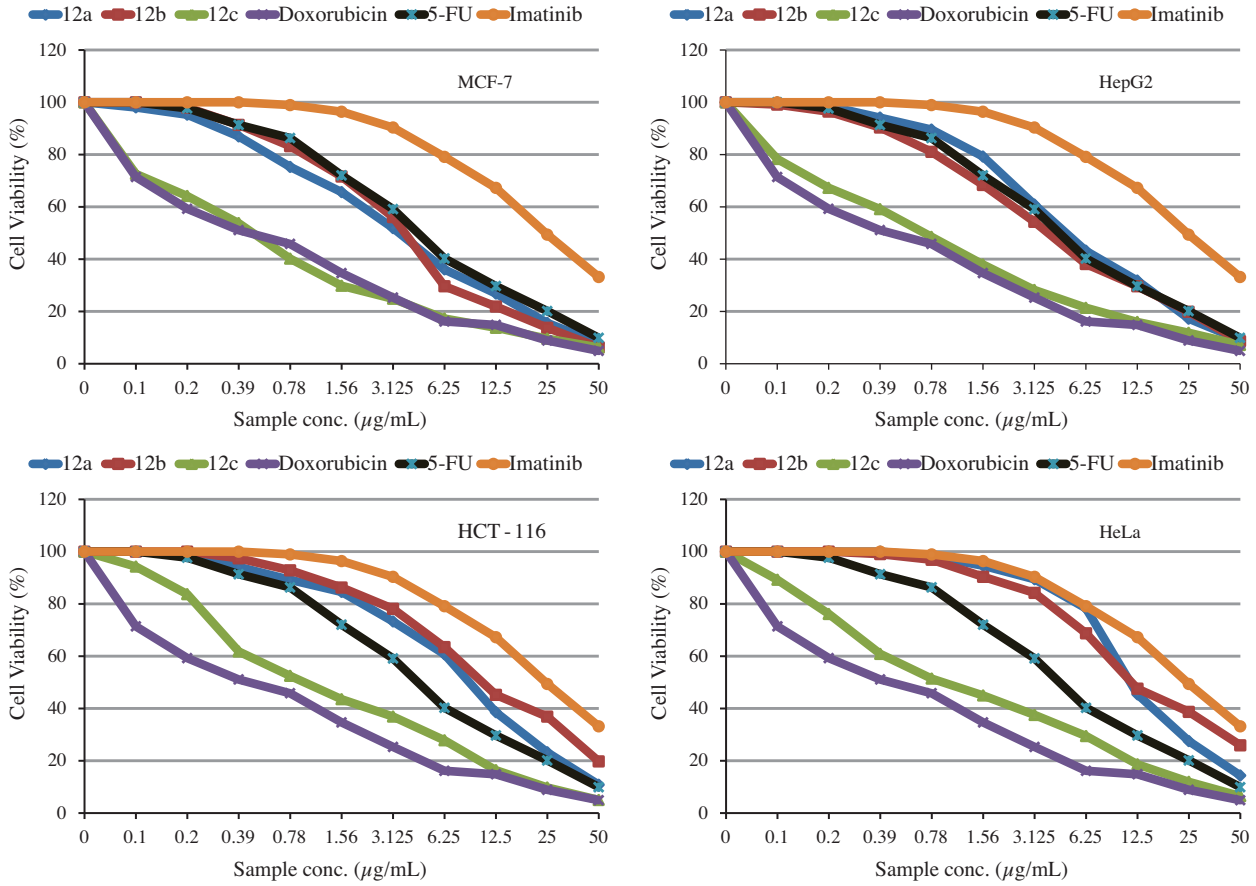


Figure 4. The dose response curve showing the in vitro inhibitory activity of compounds **12a**–**12c** compared with reference drugs against (A) breast carcinoma (MCF-7), (B) hepatocellular carcinoma, (C) colon carcinoma, and (D) human cervical carcinoma cell lines.

4. Experimental

4.1. Chemistry

Melting points were recorded on Gallenkamp electrothermal apparatus. ^{1}H NMR was determined on a Varian Gemini 300 spectrometer (300 MHz) in DMSO-d_6 with TMS as internal standard. Elemental analyses were carried out at the Microanalytical Center, University of Cairo, Giza, Egypt. Mass spectra were recorded on a GCMS-QP 1000 EX Shimadzu spectrometer. Infrared spectra (KBr) were determined on a Pye Unicam SP-3000 infrared spectrophotometer. Hydrazonoyl halides **5**[43–46] were prepared according to literature procedures.

Synthesis of 3-amino-4,6-di(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (2). A mixture of 2-mercapto-4,6-di(4-methoxyphenyl)nicotinonitrile **1** (3.84 g, 10 mmol) and potassium hydroxide (0.56 g, 10 mmol) in ethanol (20 mL) was refluxed for 8 h under reflux. The appropriate amount of 2-chloroacetamide (0.93, 10 mmol) was added and stirring was continued for 2 h. The resulting solid was collected and recrystallized from ethanol to give **2** as yellow crystals in 72% yield, mp 224 °C; IR (KBr): $\bar{V}$ = 3428, 3328, 3263, 3173 ($2NH_2$), 1654 (C=O), 1593 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.86 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 5.40 (s, D_2O exchangeable, 2H, NH_2), 5.98 (s, D_2O exchangeable, 2H,

NH_2),7.01–8.09 (m, 9H, Ar-H and pyridine-H5); MS (70 eV): m/z = 405 (M^+, 100), 180 (38), 86 (72), 58 (13). Anal. Calcd. for $C_{22}H_{19}N_3O_3S$ (405.47): C, 65.17; H, 4.72; N, 10.36. Found: C, 65.11; H, 4.75; N, 10.18%.

Synthesis of 7,9-di(4-methoxyphenyl)-2-thioxo-2,3-dihydropyrido[3',2':4,5]thieno [3,2-d]pyrimidin-4(1*H*)-one (3). To a stirred cold solution of 3-amino-4,6-di(4-methoxyphenyl)thieno[2,3-*b*]pyridine-2-carboxamide **2** (4.05 g, 10 mmol) in 30 mL of pyridine was added 30 mL of CS_2. The mixture was heated under reflux for 12 h and then evaporated under vacuum. The remaining product was poured into acidified cold water, and then the solid obtained was collected by filtration, dried, and recrystallized from DMF to give compound **3** as yellow crystals in 74% yield, mp 346–348 °C; IR (KBr): $\bar{V}$ = 3459, 3359 (2NH), 1691 (C=O), 1600 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.84 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 7.05–8.28 (m, 8H, Ar-H), 7.97 (s, 1H, pyridine-H5), 9.29 (s, D_2O exchangeable, 1H, NH), 12.99 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 448 (M^+ + 1, 18), 447 (M^+, 100), 415 (99), 247 (22), 76 (25). Anal. Calcd. for $C_{23}H_{17}N_3O_3S_2$ (447.07): C, 61.73; H, 3.83; N, 9.39. Found: C, 61.54; H, 3.67; N, 9.15%.

Synthesis of 7,9-di(4-methoxyphenyl)-2-(methylthio)pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(3*H*)-one (4). Methyl iodide (1.42 g, 10 mmol) was added to solution of thione **3** (4.47 g, 10 mmol) in 20 mL of DMF containing anhydrous K_2CO_3 (2.07 g, 15 mmol). The reaction mixture was stirred at room temperature for 4 h and then poured onto an ice-water mixture. The solid formed was filtered, washed with water, dried, and crystallized from DMF to give compound **4** as white crystals in 82% yield, mp 268 °C; IR (KBr): $\bar{V}$ = 3436 (NH), 1663 (C=O), 1605 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.56 (s, 3H, SCH_3), 3.82 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 7.05–8.24 (m, 8H, Ar-H), 7.85 (s, 1H, pyridine-H5), 12.96 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 462 (M^+ + 1, 14), 461 (M^+, 100), 178 (82), 151 (27), 76 (87). Anal. Calcd. for $C_{24}H_{19}N_3O_3S_2$ (461.56): C, 62.45; H, 4.15; N, 9.10. Found: C, 62.31; H, 4.10; N, 8.87%.

4.1.1. Synthesis of 8,10-di(4-methoxyphenyl)-1,3-disubstitutedpyrido[3',2':4,5]thieno [3,2-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1*H*)-ones (8a–l).

Method A: General procedure: triethylamine (1.4 mL, 10 mmol) was added to a mixture of equimolar amounts of thione **3** (0.447 g, 1 mmol) and the appropriate hydrazonoyl halides **5a**–**l** (10 mmol) in 50 mL of dioxane. The reaction mixture was refluxed until all of the starting materials disappeared and hydrogen sulfide gas ceased to evolve (6–10 h, monitored by TLC). The solvent was evaporated and the solid that formed was filtered and recrystallized from the appropriate solvent to give compounds **8a**–**l**, respectively.

Method B: When the above procedure was repeated using methylthio derivative **4** (0.461 g, 1 mmol) in lieu of **3**, the product proved to be identical in all respects with **8a**–**l** prepared above. The physical constants of compounds **8a**–**l** are listed below.

8,10-Di(4-methoxyphenyl)-1,3-diphenylpyrido[3',2':4,5]thieno[3,2-d][1,2,4] triazolo[4,3-a] pyrimidin-5(1*H*)-one (8a). Yellow solid, mp 296–298 °C (Dioxane); yield 78%; IR (KBr): $\bar{V}$ = 1693 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.99–8.15 (m, 18H, Ar-H), 7.78 (s, 1H, pyridine-H5); ^{13}C NMR (300 MHz, DMSO-d_6): δ = 54.6, 55.6 ($2OCH_3$), 113.3, 114.5, 119.1, 120.4, 122.4, 124.0, 124.9, 125.1, 125.7, 127.8, 128.2, 128.4, 130.2, 131.4, 132.1, 132.4, 135.3, 135.7, 137.3, 138.7, 139.7, 146.5, 148.3, 151.7, 154.5 (Ar-C), 161.6 (C=O) ppm; MS (70 eV): m/z = 608 (M^+ + 1,

64), 607 (M^+, 100), 530 (29), 302 (26), 161 (65), 77 (73). Anal. Calcd. for $C_{36}H_{25}N_5O_3S$ (607.68): C, 71.15; H, 4.15; N, 11.52. Found: C, 71.05; H, 4.11; N, 11.38%.

3-Acetyl-8,10-di(4-methoxyphenyl)-1-phenylpyrido[3',2':4,5]thieno[3,2-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1*H*)-one (8b). Yellow solid, mp 292–294 °C (DMF); yield 82%; IR (KBr): $\bar{V}$ = 1720, 1689 (2C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.75 (s, 3H, $COCH_3$), 3.76 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.99–8.19 (m, 13H, Ar-H), 7.78 (s, 1H, pyridine-H5); MS (70 eV): m/z = 574 (M^+ + 1, 28), 573 (M^+, 100), 531 (14), 286 (12), 55 (23). Anal. Calcd. for $C_{32}H_{23}N_5O_4S$ (573.62): C, 67.00; H, 4.04; N, 12.21. Found: C, 67.13; H, 3.97; N, 12.01%.

3-Acetyl-8,10-di(4-methoxyphenyl)-1-(p-tolyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo [4,3-a]pyrimidin-5(1*H*)-one (8c). Yellow solid, mp 304 °C (DMF); yield 77%; IR (KBr): $\bar{V}$ = 1720, 1689 (2C=O), 1609 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.35 (s, 3H, CH_3), 2.75 (s, 3H, $COCH_3$), 3.83 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 7.07–8.25 (m, 12H, Ar-H), 7.84 (s, 1H, pyridine-H5); MS (70 eV): m/z = 587 (M^+, 100), 545 (20), 91 (14), 77 (12). Anal. Calcd. for $C_{33}H_{25}N_5O_4S$ (587.65): C, 67.45; H, 4.29; N, 11.92. Found: C, 67.27; H, 4.20; N, 11.76%.

3-Acetyl-1-(4-chlorophenyl)-8,10-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1*H*)-one (8d). Yellow solid, mp 310–312 °C (DMF); yield 82%; IR (KBr): $\bar{V}$ = 1724, 1655 (2C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.75 (s, 3H, $COCH_3$), 3.80 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 7.07–8.23 (m, 12H, Ar-H), 7.85 (s, 1H, pyridine-H5); MS (70 eV): m/z = 609 (M^+ + 2, 43), 607 (M^+, 100), 565 (18), 304 (14), 58 (12). Anal. Calcd. for $C_{32}H_{22}ClN_5O_4S$ (608.07): C, 63.21; H, 3.65; N, 11.52. Found: C, 63.19; H, 3.53; N, 11.36%.

3-Acetyl-8,10-di(4-methoxyphenyl)-1-(4-nitrophenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidin-5(1*H*)-one (8e). Brown solid, mp 348 °C (DMF); yield 78%; IR (KBr): $\bar{V}$ = 1701, 1658 (2C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.70 (s, 3H, $COCH_3$), 3.83 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 7.03–8.20 (m, 12H, Ar-H), 7.87 (s, 1H, pyridine-H5); MS (70 eV): m/z = 618 (M^+, 7), 401 (91), 298 (100), 69 (95). Anal. Calcd. for $C_{32}H_{22}N_6O_6S$ (618.62): C, 62.13; H, 3.58; N, 13.59. Found: C, 62.06; H, 3.50; N, 13.35%.

Ethyl 8,10-di(4-methoxyphenyl)-5-oxo-1-phenyl-1,5-dihydropyrido[3',2':4,5]thieno [3,2-d] [1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8f). Yellow solid, mp 262–264 °C (Dioxane); yield 80%; IR (KBr): $\bar{V}$ = 1743, 1697 (2C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.37 (t, J= 6.7 Hz, 3H, CH_3CH_2), 3.81 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.43 (q, J= 6.7 Hz, 2H, CH_2CH_3), 7.05–8.24 (m, 13H, Ar-H), 7.82 (s, 1H, pyridine-H5); MS (70 eV): m/z = 603 (M^+, 7), 460 (61), 359 (20), 196 (25), 92 (100). Anal. Calcd. for $C_{33}H_{25}N_5O_5S$ (603.65): C, 65.66; H, 4.17; N, 11.60. Found: C, 65.48; H, 4.11; N, 11.43%.

Ethyl 8,10-di(4-methoxyphenyl)-5-oxo-1-(p-tolyl)-1,5-dihydropyrido[3',2':4,5] thieno[3,2-d] [1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8g). Pale yellow solid, mp 298–300 °C; yield 80%; IR (KBr): $\bar{V}$ = 1751, 1693 (2C=O), 1604 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.38 (t, J= 6.9 Hz, 3H, CH_3CH_2), 2.36 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.52 (q, J= 6.9 Hz, 2H, CH_2CH_3), 7.00–8.23 (m, 12H, Ar-H), 7.82 (s, 1H, pyridine-H5); MS (70 eV): m/z = 618 (M^+ + 1, 41), 617 (M^+, 100), 544 (25), 386 (11), 91(22). Anal. Calcd. for $C_{34}H_{27}N_5O_5S$ (617.67): C, 66.11; H, 4.41; N, 11.34. Found: C, 71.03; H, 4.12; N, 11.42%.

Ethyl 1,8,10-tri(4-methoxyphenyl)-5-oxo-1,5-dihydropyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8h). Yellow solid, mp 284–286 °C (Dioxane); yield 80%; IR (KBr): $\bar{V}$ = 1755, 1697 (2C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.49 (t, J= 6.9 Hz, 3H, CH_3CH_2), 3.70 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 4.58 (q, J= 6.9 Hz, 2H, CH_2CH_3), 6.84–8.15 (m, 12H, Ar-H), 7.63 (s, 1H, pyridine-H5); MS (70 eV): m/z = 634 (M^+ + 1, 20), 633 (M^+, 100), 561 (45), 386 (23), 148 (14), 92 (72). Anal. Calcd. for $C_{34}H_{27}N_5O_6S$ (633.67): C, 64.44; H, 4.29; N, 11.05. Found: C, 64.42; H, 4.13; N, 11.01%.

Ethyl 1-(4-chlorophenyl)-8,10-di(4-methoxyphenyl)-5-oxo-1,5-dihydropyrido [3',2':4,5]thieno [3,2-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8i). Pale yellow solid, mp 296 °C (Dioxane); yield 81%; IR (KBr): $\bar{V}$ = 1743, 1701 (2C=O), 1604 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.41 (t, J= 6.7 Hz, 3H, CH_3CH_2), 3.82 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.52 (q, J= 6.7 Hz, 2H, CH_2CH_3), 6.93–8.17 (m, 12H, Ar-H), 7.76 (s, 1H, pyridine-H5); MS (70 eV): m/z = 640 (M^+ + 2, 2), 638 (M^+, 7), 415 (31), 196 (44), 105 (10), 92 (100), 65 (23). Anal. Calcd. for $C_{33}H_{24}ClN5O5S$ (638.09): C, 62.12; H, 3.79; N, 10.98. Found: C, 62.08; H, 3.70; N, 10.76%.

8,10-Di(4-methoxyphenyl)-5-oxo-N,1-diphenyl-1,5-dihydropyrido[3',2':4,5]thieno [3,2-d] [1,2,4]triazolo[4,3-a]pyrimidine-3-carboxamide (8j). Yellowish white solid, mp 348 °C (DMF); yield 80%; IR (KBr): $\bar{V}$ = 3444 (NH), 1691, 1674 (2C=O), 1603 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 7.02–8.25 (m, 18H, Ar-H), 7.86 (s, 1H, pyridine-H5), 11.30 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 650 (M^+, 18), 447 (49), 195 (38), 92 (100). Anal. Calcd. for $C_{37}H_{26}N_6O_4S$ (650.71): C, 68.29; H, 4.03; N, 12.92. Found: C, 68.13; H, 4.01; N, 12.76%.

8,10-Di(4-methoxyphenyl)-5-oxo-N-phenyl-1-(p-tolyl)-1,5-dihydropyrido[3',2':4,5] thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxamide (8k). Yellowish white solid, mp 353–355 °C (DMF); yield 84%; IR (KBr): $\bar{V}$ = 3448 (NH), 1687, 1670 (2C=O), 1603 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.36 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 7.02–8.25 (m, 17H, Ar-H), 7.86 (s, 1H, pyridine-H5), 11.30 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 665 (M^+ + 1, 85), 664 (M^+, 71), 426 (100), 311 (98), 175 (86), 56 (79). Anal. Calcd. for $C_{38}H_{28}N_6O_4S$ (664.73): C, 68.66; H, 4.25; N, 12.64. Found: C, 68.43; H, 4.15; N, 12.39%.

1-(4-Chlorophenyl)-8,10-di(4-methoxyphenyl)-5-oxo-N-phenyl-1,5-dihydropyrido [3',2':4,5] thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxamide (8l). Yellowish-white solid, mp 336–338 °C (DMF); yield 88%; IR (KBr): $\bar{V}$ = 3436 (NH), 1689, 1673 (2C=O), 1601 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 7.02–8.25 (m, 17H, Ar-H), 7.88 (s, 1H, pyridine-H5), 11.28 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 687 (M^+ + 2, 11), 685 (M^+, 54), 553 (18), 421 (93), 323 (100), 152 (94), 78 (93), 65 (51). Anal. Calcd. for $C_{37}H_{25}ClN_6O_4S$ (685.15): C, 64.86; H, 3.68; N, 12.27. Found: C, 64.59; H, 3.48; N, 12.05%.

4.1.2. Synthesis of 12a–c

General procedure: 1 mL of an aqueous solution of KOH (75%) was added to 4.47 g of **3** (10 mmol) in 50 mL of ethanol and the mixture was warmed for 10 min. The appropriate chloromethylene compound **11a–c** (10 mmol) was added to the resulting clear solution and then the reaction mixture was stirred for 12 h at room temperature. The solid that precipitated was filtered off, washed with water, dried, and crystallized from the appropriate solvent to give **12a–c**, respectively.

3-((7,9-Di(4-methoxyphenyl)-4-oxo-3,4-dihydropyrido[3',2':4,5]thieno[3,2-d]pyrimidin-2-yl) thio)pentane-2,4-dione (12a). Yellowish white solid, mp 210–212 °C (Dioxane); yield 86%; IR (KBr): $\bar{V}$ = 3444 (NH), 1683, 1651, 1643 (3C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.34 (s, 6H, 2$COCH_3$), 3.88 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 4.01 (s, 1H, CH), 6.97–8.16 (m, 8H, Ar-H), 7.26 (s, 1H, pyridine-H5), 12.18 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 545 (M^+, 2), 529 (100), 448 (55), 328 (15), 136 (15), 71 (7). Anal. Calcd. for $C_{28}H_{23}N_3O_5S_2$ (545.63): C, 61.64; H, 4.25; N, 7.70. Found: C, 61.61; H, 4.17; N, 7.53%.

Ethyl 2-((7,9-di(4-methoxyphenyl)-4-oxo-3,4-dihydropyrido[3',2':4,5]thieno[3,2-d]pyrimidin -2-yl)thio)-3-oxobutanoate (12b). Yellowish white solid, mp 180–182 °C (Dioxane/EtOH); yield 73%; IR (KBr): $\bar{V}$ = 3417 (NH), 1739, 1655, 1641 (3C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.35 (t, J= 7.2 Hz, 3H, CH_3CH_2), 2.09 (s, 3H, $COCH_3$), 3.89 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.82 (s, 1H, CH), 4.34 (q, J= 7.2 Hz, 2H, CH_2CH_3), 6.96–8.16 (m, 9H, Ar-H and pyridine-H5), 12.04 (s, D_2O exchangeable, 1H, NH), MS (70 eV): m/z = 575 (M^+, 7), 486 (100), 429 (98), 316 (78), 213 (39), 136 (43), 65 (45). Anal. Calcd. for $C_{29}H_{25}N_3O_6S_2$ (575.66): C, 60.51; H, 4.38; N, 7.30. Found: C, 60.38; H, 4.33; N, 7.15%.

2-((7,9-Di(4-methoxyphenyl)-4-oxo-3,4-dihydropyrido[3',2':4,5]thieno[3,2-d]pyrimidin-2-yl) thio)-3-oxo-N-phenylbutanamide (12c). Yellowish white solid, mp 202–204 °C (Dioxane); yield 79%; IR (KBr): $\bar{V}$ = 3412, 3363 (2NH), 1692, 1659, 1648 (3C=O), 1602 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.10 (s, 3H, $COCH_3$), 3.84 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.03 (s, 1H, CH), 6.78–8.15 (m, 14H, Ar-H and pyridine-H5), 8.84 (s, D_2O exchangeable, 1H, NH), 10.39 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 622 (M^+, 7), 617 (100), 441 (43), 262 (79), 164 (21), 59 (90). Anal. Calcd. for $C_{33}H_{26}N_4O_5S_2$ (622.71): C, 63.65; H, 4.21; N, 9.00. Found: C, 63.45; H, 4.13; N, 8.87%.

4.1.3. Alternate synthesis of 8b, 8f, and 8j

A cold solution of benzenediazonium chloride, prepared by diazotizing aniline (1 mmol) dissolved in hydrochloric acid (6 M, 1 mL) with a solution of sodium nitrite (0.07 g, 1 mmol) in water (2 mL) was added portion-wise to a solution of the appropriate **12a**–**c** (1 mmol) in ethanol (20 mL) containing sodium acetate trihydrate (0.138 g, 1 mmol) while stirring and keeping the temperature below 5 °C. The reaction mixture was left for 3 h in a refrigerator. The solid precipitated was filtered off, washed with water, dried, and crystallized from DMF to give compounds **8b**, **8f**, and **8j**, which were identical in all respects (mp, mixed mp, and IR spectra) with those obtained from reaction of **3** with **5b**, **5f**, and **5j**, respectively.

Synthesis of 2-hydrazinyl-7,9-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(3*H*)-one (13). Hydrazine hydrate (80%, 20 mL) was added to 7,9-di(4-methoxyphenyl)-2-(methylthio)pyrido [3',2':4,5]thieno[3,2-d]pyrimidin-4(3*H*)-one **4** (4.61 g, 10 mmol) in dry EtOH (40 mL) and the reaction mixture was kept under reflux for 10 h and then cooled. The solid that precipitated was filtered off and crystallized from DMF to give **13** as canary yellow solid, 70% yield, mp 320–322 °C; IR (KBr): $\bar{V}$ = 3448, 3348, 3301, 3220 (NH_2 and 2NH), 1678 (C=O), 1652 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.90 (s, D_2O exchangeable, 1H, NH), 3.36 (s, D_2O exchangeable, 2H, NH_2), 3.73 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 7.02–8.22 (m, 8H, Ar-H), 7.76 (s, 1H, pyridine-H5), 8.12 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 445 (M^+, 7), 415 (7), 302 (4), 196 (64), 92 (100), 66 (31); Anal. Calcd. for $C_{23}H_{19}N_5O_3S$ (445.49): C, 62.01; H, 4.30; N, 15.72%. Found: C, 62.24; H, 4.13; N, 15.48%.

4.1.4. Synthesis of hydrazones 15a–f

A mixture of hydrazine **13** (0.89 g, 2 mmol) and the appropriate aldehyde **14a–f** (2 mmol) in acetic acid (20 mL) and a few drops of conc. hydrochloric acid (≈1 mL) was heated under reflux for 5 h. The reaction mixture was then cooled and diluted with water. The so-formed solid product was then collected by filtration, dried, and recrystallized from DMF to afford the corresponding hydrazones **15a–f**.

2-(2-Benzylidenehydrazinyl)-7,9-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d] pyrimidin-4(3*H*)-one (15a). Yellow solid, mp 346–348 °C; yield 74%; IR (KBr): $\bar{V}$ = 3444, 3359 (2NH), 1658 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.84 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 7.06–8.25 (m, 13H, Ar-H), 7.92 (s, 1H, pyridine-H5), 8.17 (s, 1H, =CH), 11.11, 11.79 (2s, D_2O exchangeable, 2H, 2NH); MS (70 eV): m/z = 533 (M^+, 61), 430 (54), 387 (26), 301 (14), 228 (12),104 (100), 77 (69). Anal. Calcd. for $C_{30}H_{23}N_5O_3S$ (533.60) C, 67.53; H, 4.34; N, 13.12. Found: C, 67.39; H, 4.37; N, 13.04%.

2-(2-(4-Chlorobenzylidene)hydrazinyl)-7,9-di(4-methoxyphenyl)pyrido[3',2':4,5] thieno[3,2-d]pyrimidin-4(3*H*)-one (15b). Yellow solid, mp 352–354 °C; yield 73%; IR (KBr): $\bar{V}$ = 3448, 3355 (2NH), 1651 (C=O), 1604 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 7.04–8.22 (m, 12H, Ar-H), 7.78 (s, 1H, pyridine-H5), 8.13 (s, 1H, =CH), 11.16, 11.86 (2s, D_2O exchangeable, 2H, 2NH); MS (70 eV): m/z = 569 (M^+ + 2, 20), 567 (M^+, 38), 456 (50), 359 (19), 275 (16), 137 (64), 104 (12), 73 (100). Anal. Calcd. for $C_{30}H_{22}ClN_5O_3S$ (568.05) C, 63.43; H, 3.90; N, 12.33. Found: C, 63.26; H, 3.76; N, 12.16%.

2-(2-(4-Methoxybenzylidene)hydrazinyl)-7,9-bis(4-methoxyphenyl)pyrido[3',2':4,5] thieno [3,2-d]pyrimidin-4(3*H*)-one (15c). Yellow solid, mp 324–326 °C; yield 76%; IR (KBr): $\bar{V}$ = 3444, 3348 (2NH), 1662 (C=O), 1604 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.80 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.95–8.25 (m, 12H, Ar-H), 7.80 (s, 1H, pyridine-H5), 8.11 (s, 1H, =CH), 10.98, 11.69 (2s, D_2O exchangeable, 2H, 2NH); MS (70 eV): m/z = 563 (M^+, 18), 415 (100), 359 (24), 273 (14), 135 (26), 99 (16), 75 (16). Anal. Calcd. for $C_{31}H_{25}N_5O_4S$ (563.63) C, 66.06; H, 4.47; N, 12.43. Found: C, 66.25; H, 4.30; N, 12.23%.

7,9-Di(4-methoxyphenyl)-2-(2-(4-nitrobenzylidene)hydrazinyl)pyrido[3',2':4,5] thieno[3,2-d] pyrimidin-4(3*H*)-one (15d). Orange solid, mp 350 °C; yield 73%; IR (KBr): $\bar{V}$ = 3448, 3363 (2NH), 1670 (C=O), 1604 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.81 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.98–8.23 (m, 12H, Ar-H), 7.86 (s, 1H, pyridine-H5), 8.19 (s, 1H, =CH), 11.08, 11.81 (2s, D_2O exchangeable, 2H, 2NH); MS (70 eV): m/z = 578 (M^+, 1), 447 (79), 330 (6), 287 (43), 121 (66), 92 (32), 58 (100). Anal. Calcd. for $C_{30}H_{22}N_6O_5S$ (578.60) C, 62.27; H, 3.83; N, 14.52. Found: C, 62.20; H, 3.64; N, 14.31%.

2-(2-(2-Hydroxybenzylidene)hydrazinyl)-7,9-bis(4-methoxyphenyl)pyrido[3',2':4,5] thieno [3,2-d]pyrimidin-4(3*H*)-one (15e). Yellow solid, mp 331–333 °C; yield 74%; IR (KBr): $\bar{V}$ = 3506–3444, 3375 (OH and 2NH), 1658 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.84 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.84–8.24 (m, 12H, Ar-H), 7.80 (s, 1H, pyridine-H5), 8.49 (s, 1H, =CH), 9.88, 11.12 (2s, D_2O exchangeable, 2H, 2NH), 11.90 (1s, 1H, D_2O exchangeable, OH); MS (70 eV): m/z = 549 (M^+, 17), 359 (19), 274 (8), 120 (44), 85 (98), 57 (100). Anal. Calcd. for $C_{30}H_{23}N_5O_4S$ (549.60) C, 65.56; H, 4.22; N, 12.74. Found: C, 65.50; H, 4.16; N, 12.53%.

2-(2-(2,4-Dichlorobenzylidene)hydrazinyl)-7,9-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno [3,2-d]pyrimidin-4(3H)-one (15f). Yellow solid, mp 344–345 °C; yield 78%; IR (KBr): $\bar{V}$ = 3448, 3348 (2NH), 1662 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.78 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 7.18–8.24 (m, 11H, Ar-H), 7.88 (s, 1H, pyridine-H5), 8.20 (s, 1H, =CH), 11.13, 11.89 (2s, D_2O exchangeable, 2H, 2NH); MS (70 eV): m/z = 603 (M^+, 2), 447 (100), 430 (45), 315 (39), 121 (47), 59 (77). Anal. Calcd. for $C_{30}H_{21}Cl_2N_5O_3S$ (602.49) C, 59.81; H, 3.51; N, 11.62. Found: C, 59.79; H, 3.48; N, 11.50%.

4.1.5. Cyclization of hydrazones 15a–f

Bromine (0.052 g, 1 mmol) in acetic acid (5 mL) was added dropwise to a stirred solution of the appropriate hydrazone **15a–f** (1 mmol of each) in acetic acid (10 mL) and sodium acetate (0.5 g). The reaction mixture was then poured onto ice cold water, and the solid that precipitated was filtered off, washed with sodium bicarbonate solution and then with water, dried, and crystallized from DMF to give the respective compounds **16a–f.**

8,10-Di(4-methoxyphenyl)-3-phenylpyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo [4,3-a] pyri= midin-5(1*H*)-one (16a). White solid, mp 320 °C; yield 69%; IR (KBr): $\bar{V}$ = 3434 (NH), 1689 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.82 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.63–8.28 (m, 13H, Ar-H), 7.84 (s, 1H, pyridine-H5), 11.76 (s, D_2O exchangeable, 1H, NH); ^{13}C NMR (300 MHz, DMSO-d_6): δ = 54.3, 55.2 ($2OCH_3$), 114.9, 117.5, 119.1, 122.1, 122.6, 124.9, 125.1, 125.7, 127.8, 129.1, 131.4, 132.1, 133.7, 135.3, 135.7, 137.3, 138.7, 139.9, 153.6, 154.1, 156.5 (Ar-C), 162.0 (C=O) ppm; MS (70 eV): m/z = 531 (M^+, 26), 432 (100), 415 (92), 388 (27), 273 (6), 180 (43), 64 (59). Anal. Calcd. for $C_{30}H_{21}N_5O_3S$ (531.58) C, 67.78; H, 3.98; N, 13.17. Found: C, 67.57; H, 3.79; N, 13.03%.

3-(4-Chlorophenyl)-8,10-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1*H*)-one (16b). Yellow solid, mp 330–332 °C; yield 68%; IR (KBr): $\bar{V}$ = 3432 (NH), 1688 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.83 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 7.12–8.28 (m, 12H, Ar-H), 7.83 (s, 1H, pyridine-H5), 11.87 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 568 (M^+ + 2, 31), 566 (M^+, 98), 432 (7), 398 (20), 250 (9), 120 (40), 92 (41), 64 (100). Anal. Calcd. for $C_{30}H_{20}ClN_5O_3S$ (566.03) C, 63.66; H, 3.56; N, 12.37. Found: C, 63.51; H, 3.44; N, 12.25%.

3,8,10-Tri(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo[4,3-a]pyrimidin -5 (1*H*)-one (16c). White solid, mp 318–319 °C; yield 68%; IR (KBr): $\bar{V}$ = 3433 (NH), 1686 (C=O), 1607 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.78 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.74–8.29 (m, 12H, Ar-H), 7.89 (s, 1H, pyridine-H5), 11.67 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 561 (M^+, 4), 432 (100), 415 (94), 388 (26), 135 (21), 64 (67). Anal. Calcd. for $C_{31}H_{23}N_5O_4S$ (561.61) C, 66.30; H, 4.13; N, 12.47. Found: C, 66.16; H, 4.02; N, 12.40%.

8,10-Di(4-methoxyphenyl)-3-(4-nitrophenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1*H*)-one (16d). Brown solid, mp 347–349 °C; yield 71%; IR (KBr): $\bar{V}$ = 3434 (NH), 1693 (C=O), 1606 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.79 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.72–8.28 (m, 12H, Ar-H), 7.81 (s, 1H, pyridine-H5), 11.82 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 576 (M^+, 5), 432 (57), 388 (6), 135 (11), 92 (12), 64 (100). Anal. Calcd. for $C_{30}H_{20}N_6O_5S$ (576.58) C, 62.49; H, 3.50; N, 14.58. Found: C, 62.42; H, 3.31; N, 14.46%.

3-(2-Hydroxyphenyl)-8,10-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4] triazolo [4,3-a]pyrimidin-5(1*H*)-one (16e). Yellow solid, mp 352–353 °C; yield 71%; IR (KBr): $\bar{V}$ = 3519–3400

(OH and NH), 1673 (C=O), 1606 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.80 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.68–8.24 (m, 12H, Ar-H), 7.81 (s, 1H, pyridine-H5), 11.12 (s, D_2O exchangeable, 1H, NH), 11.83 (s, 1H, D_2O exchangeable, OH); MS (70 eV): m/z = 547 (M^+, 3), 432 (100), 388 (24), 137 (15), 60 (91). Anal. Calcd. for $C_{30}H_{21}N_5O_4S$ (547.58) C, 65.80; H, 3.87; N, 12.79. Found: C, 65.59; H, 3.81; N, 12.58%.

3-(2,4-Dichlorophenyl)-8,10-di(4-methoxyphenyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,4]triazolo [4,3-a]pyrimidin-5(1*H*)-one (16f). White solid, mp 316–318 °C; yield 72%; IR (KBr): $\bar{V}$ = 3433 (NH), 1691 (C=O), 1605 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.80 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 6.67–8.33 (m, 11H, Ar-H), 7.89 (s, 1H, pyridine-H5), 11.74 (s, D_2O exchangeable, 1H, NH); MS (70 eV): m/z = 601 (M^+, 44), 494 (48), 432 (100), 387 (29), 136 (45), 64 (76). Anal. Calcd. for $C_{30}H_{19}Cl_2N_5O_3S$ (600.47) C, 60.01; H, 3.19; N, 11.66. Found: C, 59.89; H, 3.04; N, 11.47%.

4.1.6. Alternate synthesis of 16a

To a solution of hydrazine **13** (0.445 g, 1 mmol) in dry pyridine (20 mL) was added benzoyl chloride (0.14 g, 1 mmol) and the resulting mixture was refluxed for 2 h. After cooling, the precipitate was collected by filtration and crystallized from DMF to afford a product that was found to be identical in all respects (mp, mixed mp, and IR) with product **16a**.

4.2. Biological evaluation

4.2.1. Antimicrobial activity assay

All microbial strains were provided from the culture collection of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The preliminary antimicrobial activity was investigated on a dozen newly synthesized compounds in order to increase the selectivity of these derivatives towards test microorganisms. Briefly, 100 μL of the test bacteria/fungi were grown in 10 mL of fresh media until they reached a count of approximately 10^8 cells/mL for bacteria or 10^5 cells/mL for fungi.[47] Then 100 μL of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained and tested for susceptibility by the well diffusion method. The National Committee for Clinical Laboratory Standards (NCCLS) recommends Mueller-Hinton and Sabouraud agar as they result in good batch-to-batch reproducibility. The diffusion method for filamentous fungi and yeast was tested by using the approved standard methods (M38-A and M44-P, respectively) developed by the NCCLS[48] for evaluating susceptibilities to antifungal agents. One hundred microliters of each sample (at 5 mg/mL) was added to each well (10 mm diameter holes cut in the agar gel). The plates were incubated for 24–48 h at 37 °C (for bacteria and yeast) and for 48 h at 28 °C (for filamentous fungi). After incubation, the microorganism's growth was observed. The resulting inhibition zone diameters were measured in millimeters and used as the criterion for antimicrobial activity. If an organism is placed on the agar it will not grow in the area around the well if it is susceptible to the chemical. This area of no growth around the disk is known as a zone of inhibition. The size of the clear zone is proportional to the inhibitory action of the compound under investigation. Solvent controls (DMSO) were included in every experiment as negative controls. DMSO was used for dissolving the tested compounds and showed no inhibition zones, confirming that it has no influence on growth of the tested microorganisms.

4.2.2. MIC determination using the broth microdilution method

The in vitro antimicrobial activity of the synthesized compounds was screened using the broth dilution method as described by CLSI[49] to determine the lowest concentration inhibiting growth of the organism recorded as the MIC using DMSO as diluent. Mueller-Hinton broth was used as nutrient medium to grow and dilute the drug suspension for the tested bacteria, and Sabouraud dextrose broth was used for fungal nutrition. The stock 1000 μg/mL was prepared. For the broth microdilution test, 50 μL of each microbial suspension in suitable growth medium was added to the wells of a sterile 96-well microtiter plate already containing 50 μL of two-fold serially diluted tested compound. Control wells were prepared with culture medium, microbial suspension only, tested compound only, and DMSO in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 min prior to incubation for 24–48 h in the cultivation conditions described above. The MIC was the lowest concentration where no viability was observed after 24–48 h on the basis of metabolic activity. To indicate respiratory activity the presence of color was determined after adding 10 μL/well of 2,3,5-triphenyl tetrazolium chloride dissolved in water (20 mg/mL) and incubation under appropriate cultivation conditions for 30 min in the dark.[50] After incubation, the optical density was measured by a microplate reader. Positive controls were wells with a microbial suspension in an appropriate growth medium in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and tested compound. All measurements of MIC values were repeated in triplicate. Tetracycline and ampicillin were used as standard antibacterial drugs, while griseofulvin and amphotericin B were used as standard antifungal drugs.

4.2.3. Evaluation of the antitumor activity using a viability assay

All human anticancer cell lines were obtained from the American Type Culture Collection. The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μg/mL gentamicin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

Gangadevi and Muthumary's method[51] was used for evaluation of the potential cytotoxicity of the tested compounds. The number of surviving cells was determined by staining the cells with crystal violet[51,52] followed by cell lysing using 33% glacial acetic acid and reading the absorbance at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) after mixing well.

The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

5. Conclusion

A novel series of fused [1,2,4]triazolo[1,5-*a*]pyrimidines were synthesized by different methods and evaluated for their in vitro antibacterial, antifungal, and anticancer activities. From the screening results, it can be seen that compounds **8f** and **15f** showed excellent activity against gram-positive bacteria and compound **15d** showed potent activity against gram-negative bacteria. The rest of the compounds showed good or moderate activity against the tested bacteria compared with the standard drugs. Compounds **8d**, **8e**, **8c**, **8l**, and **8j** among the compounds tested exhibited higher antifungal activity as compared with the reference drug against yeast species. The in vitro cytotoxic activity testing revealed that compounds **12c**, **12a**, and **12b** exhibited promising inhibitory activity against the four tested tumor cells (MCF-7, HepG-2, HCT-116, and HeLa).

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