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Synthesis and Biological Evaluation of Some Novel Polysubstituted Pyrimidine Derivatives as Potential Antimicrobial and Anticancer Agents*

Sherif A. F. Rostom[1], Hayam M. A. Ashour[2], and Heba A. Abd El Razik[2]

[1] Department of Chemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
[2] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

Synthesis and evaluation of the antimicrobial and cytotoxic activity of two series of polysubstituted pyrimidines comprising the thioether functionality and other pharmacophores, reported to contribute to various chemotherapeutic activities are described. All newly synthesized compounds were subjected to *in-vitro* antibacterial and antifungal screening. Out of the compounds tested, 18 derivatives displayed an obvious inhibitory effect on the growth of the tested Gram-positive and Gram-negative bacterial strains, with special effectiveness against the Gram-positive strains. Compounds **1**, **2**, **6**, **7**, **9**, **10**, **11**, **21**, and **24** revealed remarkable broad antibacterial spectrum profiles. Among those, compounds **1**, **2**, **6**, **7**, **9**, and **24** exhibited an appreciable antifungal activity against *C. albicans*. Compound **2** proved to be the most active antimicrobial member identified here as it showed twice the activity of ampicillin against *B. subtilis* and the same activity of ampicillin against *M. Luteus* and *P. aeruginosa* together with a moderate antifungal activity. Further, eleven analogs were evaluated for their *in-vitro* cytotoxic potential utilizing the standard MTT assay against a panel of three human cell lines: breast adenocarcinoma MCF7, hepatocellular carcinoma HePG2, and colon carcinoma HT29. The obtained data revealed that six of the tested compounds **1**, **3**, **7**, **12**, **13**, and **15** showed a variable degree of cytotoxic activity against the tested cell lines at both the LC_{50} and LC_{90} levels. Compound **7** proved to be the most active cytotoxic member in this study with special effectiveness against the colon carcinoma HT29 and breast cancer MCF7 human cell lines for LC_{50} and LC_{90}. Thus, compounds **1** and **7** could be considered as possible dual antimicrobial-anticancer agents.

Keywords: Antibacterial / Anticancer activity / Antifungal / Pyrimidines / Thioether

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Introduction

Over the past two decades, the health benefits ascribed to commercially available antimicrobials became doubtful, since many commonly used antibiotics have become less effective against certain bacterial infections, not only because of the toxic reactions they produce, but also due to emergence of drug resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) [1, 2]. Moreover, there has been a rapid spread in primary and opportunistic fungal infections particularly *Candida albicans*, because of the increased number of immuno-compromised patients suffering from AIDS, cancer, and organ transplantation [3, 4]. Consequently, such types of infections continue to provide impetus for the search and discovery of novel, more potent, and selective non-traditional anti-

Correspondence: Sherif A. F. Rostom, Ph.D., Department of Chemistry, Faculty of Science, King Abdulaziz University, PO Box 80203, Jeddah 21589, Saudi Arabia.
E-mail: sherifrostom@yahoo.com
Fax: +9662-6400000-22327

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microbial agents so that no cross-resistance with the present therapeuticals can take place.

On the other hand, the increasing number of neoplastic diseases together with the accompanied high mortality rates [5] has stimulated an unprecedented level of research directed towards the search for new structure leads that might be of use in designing novel antitumor drugs.

Patients with neoplastic disorders that are subjected to chemotherapeutic treatment are highly susceptible to microbial infections due to subsequent lack of immunity. Co-administration of multiple drugs for treating patients suffering from cancer disease accompanied with microbial infections might inflect some added health problems especially in patients with impaired liver and / or kidney functions. Therefore, the concept of monotherapy by a single drug which possesses dual utility might be advantageous from both therapeutic and cost-effective stand points.

Among the pharmacologically most interesting chemical scaffolds, are pyrimidines, widely spread in many natural products and in several interesting nucleoside and non-nucleoside compounds. Being a building unit of DNA and RNA, pyrimidine derivatives were found to be associated with a variety of chemotherapeutic effects including antimicrobial [6–10], antitubercular [11], antifungal [12], antimalarial [13], and antiviral [14, 15] activities. Furthermore, pyrimidine-containing compounds were reported to contribute to a variety of anticancer potentials including antitumor [16], antineoplastic [17], antiproliferative [18, 19], thymidylate synthase and dihydrofolate reductase inhibitory [20], angiogenesis inhibiting [21], and cyclin-dependent kinase inhibitory [22] activities. Interest in the chemotherapeutic activity of pyrimidines stemmed from the early success of some pyrimidine-based antimetabolites such as 5-flurouracil, carmofur, 5-azauracil, cytarabine, and gemcitabine as potential antineoplastic agents [23]. Diverse mechanisms of actions were reported to be encountered with the chemotherapeutic bioactivity of pyrimidines including inhibition of kinases, inhibition of enzymes involved in pyrimidine biosynthesis, incorporation into RNA and DNA which subsequently cause misreading and inhibition of DNA polymerase [23].

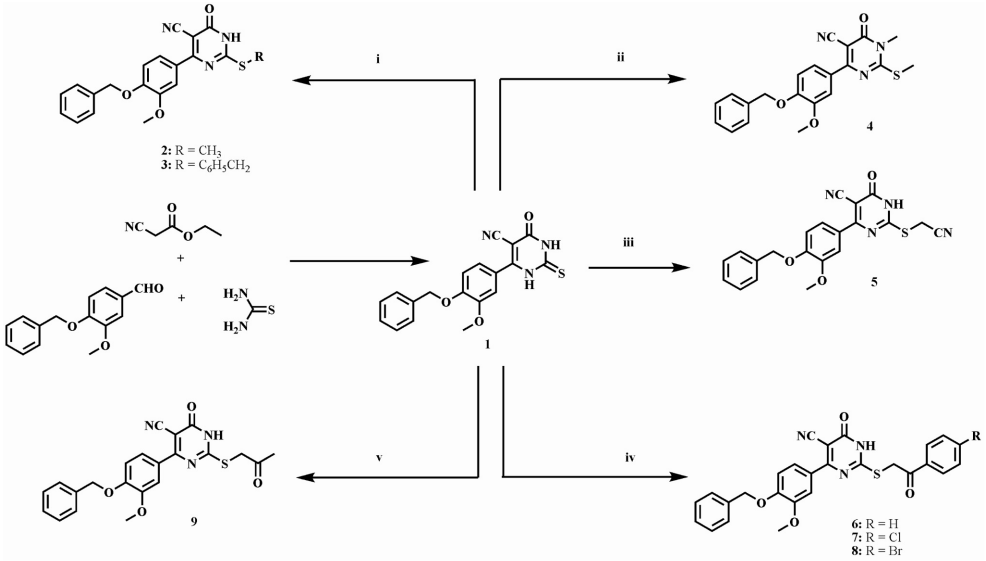
In view of the above mentioned facts, and inspired by the promising chemotherapeutic activities encountered with some pyrimidine derivatives reported in our previous publications [24–27], we considered it worthwhile to synthesize some novel polysubstituted pyrimidines with the hope of discovering more active and selective antimicrobial and / or anticancer agents.

A literature survey revealed that many pyrimidine-5-carbonitriles [28, 29] and pyrimidinethione derivatives [30] proved to exhibit potent anticancer as well as antimicrobial activities. Moreover, it has been well documented that incorporation of alkoxy substituents (methoxy and / or benzyloxy moieties) results in significant enhancement of several biological activities due to the magnification of compounds' lipophilicity [31–34]. Consequently, the newly synthesized compounds were designed so as to constitute essentially a pyrimidinethione ring system substituted basically with a cyano group and a highly lipophilic alkoxylated aryl moiety. The thrust in derivatization of such scaffold was focussed on the thione function that was linked to a variety of pharmacologically active substituents and functionalities through -CH_2- or -CH_2CO- bridges, or directly hooked to the sulfur atom to produce the target thioethers. Thioethers were found to show enhanced antimicrobial and antitumor activities [35–37], beside being analogs of *S*-DABOs (dihydro-alkylthio-benzyl-oxopyrimidines) which possess antiproliferative as well as antiviral activities [38, 39]. Moreover, at a certain stage of this investigation, the synthesis of chloropyrimidines was planned owing to their reported potential antimicrobial and antitumor activities [40, 41]. The fact that hydrazino derivatives are capable of exerting anticancer activity by alkylating DNA through a free radical intermediate [42], prompted the synthesis of some substituted amino- and hydrazinopyrimidines in order to study the influence of such structure variation on the anticipated biological activities.

Results and discussion

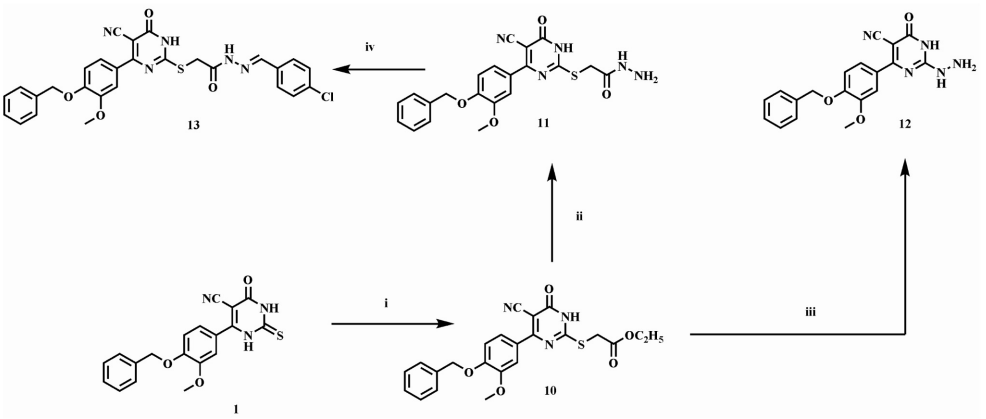
Chemistry

Synthesis of the intermediate and target compounds was accomplished according to the steps depicted in Schemes 1, 2, and 3. In Scheme 1, the key intermediate 6-(4-benzyloxy-3-methoxyphenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile **1** was prepared according to previously reported reaction conditions [43]. Stirring **1** with an equivalent amount of either methyl iodide or benzyl chloride in dry DMF containing anhydrous potassium carbonate gave rise to the *S*-alkyl derivatives **2** and **3**; respectively. However, treating **1** with three equivalents of methyl iodide under the same reaction conditions yielded the dimethyl analog **4**. Moreover, treating the thione **1** with chloroacetonitrile afforded the corresponding 2-cyanomethylsulfanyl derivative **5**. Furthermore, alkylation of the same starting compound **1** either with 4-substituted phenacyl bromide or chloroacetone



Reagents and reaction conditions: i) RX, K_2CO_3, DMF, rt.; ii) CH_3I (3 eq.), K_2CO_3, DMF, rt.; iii) $ClCH_2CN$, K_2CO_3, DMF, rt.; iv) R-$C_6H_4COCH_2Br$, K_2CO_3, DMF, rt.; v) $ClCH_2COCH_3$, K_2CO_3, DMF, rt.

Scheme 1. Synthesis of compounds **1** – **9**.



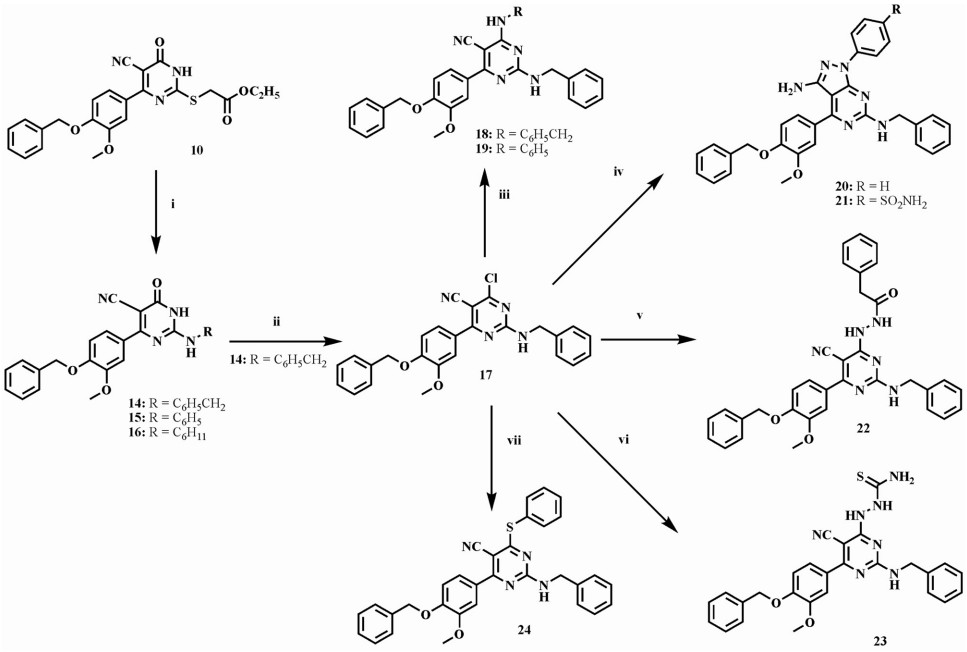
Reagents and reaction conditions: i) $ClCH_2COOC_2H_5$, K_2CO_3, DMF, rt.; ii) NH_2NH_2, EtOH, rt.; iii) NH_2NH_2, EtOH, reflux; iv) 4-Cl-C_6H_4-CHO, EtOH, reflux.

Scheme 2. Synthesis of compounds **10** – **13**.

yielded the targeted substituted phenylcarbonylmethylsulfanyl **6** – **8** and 2-oxoprop-1-ylsulfanyl **9** analogs.

On the other hand, alkylation of the thione **1** with ethyl chloroacetate afforded the substituted ethyl acetate derivative **10**. Stirring of **10** with excess hydrazine hydrate in absolute ethanol at room temperature afforded the acid hydrazide **11** in a moderate yield. However, refluxing **10** with excess hydrazine hydrate resulted in the corresponding hydrazino derivative **12**. Additionally, the azomethine **13** was prepared by condensing the acid hydrazide **11** with 4-chlorobenzaldehyde in ethanol (Scheme 2).

At this stage, it was planned to synthesize some 4-(4-benzyloxy-3-methoxyphenyl)-2-substituted amino-6-oxo-1,6-dihydropyrimidine-5-carbonitriles **14** – **16** (Scheme 3). This was achieved by fusion of the acetate ester **10** with four equivalents of the appropriate amine in an oil bath at 160 – 170°C. Furthermore, the 2-benzylamino derivative **14** was utilized for the synthesis of the chloro derivative **17** through reflux in $POCl_3$ for 15 min, since increasing the reaction time led to an increase in the percentage of by-products probably due to cleavage of the ether linkage. Refluxing **17** with aniline or benzyl amine in absolute ethanol yielded the corresponding amino deriv-



Reagents and reaction conditions: i) RNH_2, heating 160–170°C; ii) $POCl_3$, reflux, 15 min; iii) RNH_2, EtOH, reflux, 6 h; iv) 4-R-$C_6H_4NHNH_2 \cdot HCl$, anhydrous NaOAc, EtOH, reflux, 12 h; v) $C_6H_5CH_2CONHNH_2$, EtOH, reflux, 18 h; vi) $NH_2CSNHNH_2$, EtOH, reflux, 18 h; vii) C_6H_5SH, K_2CO_3, DMF, rt.

Scheme 3. Synthesis of compounds **14**–**24**.

atives **18** and **19**, respectively. However, refluxing **17** with the selected arylhydrazine hydrochloride in absolute ethanol containing anhydrous sodium acetate resulted in the formation of the pyrazolo[3,4-*d*]pyrimidines **20**, **21**. IR spectra of the latter compounds revealed the absence of the characteristic cyano group, whereas, their ^{1}H-NMR spectra displayed a D_2O-exchangeable singlet attributable to a NH_2 group. Moreover, the mass spectrum of compound **20** was in favor of the unexpected structure as it showed a molecular ion peak at m/z 528. On the other hand, refluxing **17** with phenyl acetic acid hydrazide or thiosemicarbazide in absolute ethanol yielded the requisite derivatives **22** and **23**, respectively. Finally, stirring **17** with an equivalent amount of thiophenol in dry DMF containing anhydrous potassium carbonate afforded the target 6-phenylsulfanyl derivative **24**.

In-vitro antibacterial and antifungal activities

All the newly synthesized compounds were evaluated for their *in-vitro* antibacterial activity against *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (NRRL B-14819), and *Micrococcus luteus* (ATCC 21881) as examples of Gram-positive bacteria and *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumonia* (clinical isolate) as examples of Gram-negative bacteria. They were also evaluated for their *in-vitro* antifungal potential against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (recultured) fungal strains.

The agar-diffusion method was used for determination of preliminary antibacterial and antifungal activity. Ampicillin trihydrate (antibiotic) and clotrimazole (antifungal) were used as reference drugs. The results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the discs in mm. The minimum inhibitory concentration (MIC) measurement was determined for compounds that showed significant growth inhibition zones (≥14 mm) using the two-fold serial dilution method [44]. The MIC values (in μg/mL) of the active compounds against the tested microbial strains are recorded in Table 1.

Concerning the antibacterial activity, the results revealed that 18 out of the tested 24 compounds displayed variable inhibitory effects on the growth of the tested Gram-positive and Gram-negative bacterial strains. In general, most of the tested compounds revealed better antibacterial activity against the Gram-positive rather than the Gram-negative bacteria. Among the Gram-positive bacteria tested, two strains namely, *S. aureus* and *B. subtilis*, showed relatively high sensitivity towards the tested compounds. In this view, compounds **1**, **2**, **9**, **17**, **19**, and **21** (MIC = 12.5 μg/mL) were 50% less active than

Table 1. Minimal inhibitory concentrations (MIC, in μg/mL) of the active newly synthesized compounds.

Compound	*S. aureus*	*B. subtilis*	*M. luteus*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*C. albicans*
1	12.5	25	25	50	25	100	12.5
2	12.5	6.25	12.5	12.5	12.5	50	50
3	50	50	100	100	100	100	– [a]
5	100	100	100	100	100	–	–
6	25	12.5	25	12.5	25	25	12.5
7	50	12.5	25	25	50	50	25
9	12.5	25	50	50	25	50	25
10	25	25	50	50	50	50	–
11	25	25	25	50	25	50	–
13	100	100	–	100	–	–	–
14	25	50	100	50	50	–	–
17	12.5	12.5	50	100	–	–	–
18	50	100	-	50	–	–	–
19	12.5	50	100	50	–	–	–
20	50	50	100	100	100	–	–
21	12.5	12.5	50	25	50	–	–
22	50	50	–	–	–	–	–
24	25	25	100	12.5	50	100	50
A [b]	6.25	12.5	12.5	6.25	12.5	12.5	–
C [c]	–	–	–	–	–	–	6.25

[a] (–): totally inactive (MIC ≥ 200 μg/mL).
[b] **A**: Ampicillin trihydrate (standard broad-spectrum antibiotic).
[c] **C**: Clotrimazole (standard broad-spectrum antifungal agent).

ampicillin (MIC = 6.25 μg/mL) against *S. aureus*. Moreover, the analogs **6**, **10**, **11**, **14**, and **24** (MIC = 25 μg/mL) showed 25% of the activity of ampicillin against the same organism. The rest of the active compounds revealed moderate activity against *S. aureus* (MIC range: 50 – 100 μg/mL). With regard to the activity against *B. subtilis*, an outstanding growth inhibitory activity was displayed by compound **2** (MIC = 6.25 μg/mL) which was twice as active as ampicillin (MIC = 12.5 μg/mL) against this organism. Furthermore, compounds **6**, **7**, **17**, and **21** were equipotent to ampicillin against the same organism (MIC = 12.5 μg/mL), whereas the analogs **1**, **9**, **10**, **11**, and **24** (MIC = 25 μg/mL) exhibited half the potency of ampicillin (MIC = 12.5 μg/mL) against the same species. *M. luteus* proved to be the least sensitive Gram-positive microorganism to most of the tested compounds, however, compound **2** was shown to act equipotent to ampicillin against this organism (MIC = 12.5 μg/mL). Four compounds namely, **1**, **6**, **7**, and **11** (MIC 25 μg/mL) exhibited appreciable growth inhibitory effect towards *M. luteus* which was 50% of the activity of ampicillin (MIC = 12.5 μg/mL). On the other hand, investigation of the antibacterial potency of the active compounds against the three tested Gram-negative strains revealed that three analogs namely **2**, **6**, and **24** were able to produce noticeable growth inhibitory activity against *E. coli* (MIC = 12.5 μg/mL) which represents half the activity of ampicillin (MIC = 6.25 μg/mL). Whereas, compounds **7** and **21** (MIC = 25 μg/mL) exhibited moderate activity against the same organism when compared with ampicillin (MIC = 6.25 μg/mL). Additionally, the tested *P. aeruginosa* and *K. pneumonia* strains revealed moderate to weak sensitivity to most of the active compounds (MIC range: 25 – 100 μg/mL). An exception was shown by compound **2** which was proved to be equipotent to ampicillin against *P. aeruginosa* (MIC = 12.5 μg/mL).

Concerning the antifungal activity of the tested compounds, the results revealed that only six compounds namely; **1**, **2**, **6**, **7**, **9**, and **24** were able to produce appreciable growth inhibitory activity against *C. albicans* (MIC values: 12.5 – 100 μg/mL, respectively) comparable to that of clotrimazole (MIC = 6.25 μg/mL), the standard antifungal agent utilized in this assay. Among these, the analogs **1** and **6** exhibited the best antifungal activity (MIC = 12.5 μg/mL) that is 50% of the standard. It should be noted that all of the tested compounds lacked antifungal activity against *Asp. niger*.

A deep insight into the structures of the active compounds revealed that the tested compounds belong to two main series namely; 4-(4-benzyloxy-3-methoxyphenyl)-2-substituted sulfanyl-6-oxo-1,6-dihydropyrimidine-5-carbonitriles **1** – **13** (Schemes 1 and 2) and 4-(4-benzyloxy-3-methoxy-phenyl)-2,6-disubstitutedaminopyrimidine-5-carbonitriles **14** – **24** (Scheme 3).

Within the first series (Scheme 1), the results revealed that the parent thione **1** exhibited potential activity

against all the tested microorganisms. It showed 50% of the activity of ampicillin against the Gram-positive organisms *S. aureus*, *B. subtilis*, *M. Luteus* and the Gram-negative strain *P. aeruginosa*. However, the antimicrobial potential of the derived compounds is obviously dependant on the nature of the substituent of the thio function at position 2. In this respect, alkylation of the 2-thione functionality resulted in two active compounds (**2**: R = CH_3 and **3**: R = CH_2-C_6H_5), of which the methyl analog **2** showed significant enhancement in activity especially against *B. subtilis* as the compound displayed double the activity of ampicillin against this organism. It revealed also a two-fold improvement in the antibacterial potential against *M. Luteus, P. Aeruginosa*, and *K. pneumonia* and a four-fold improvement in activity against *E. coli*. Meanwhile, it is shown to be equipotent to ampicillin against *M. Luteus* and *P. aeruginosa*. However, the benzyl analog **3** showed a dramatic reduction in the overall antimicrobial spectrum when compared with the parent thione **1**. Dimethylation at positions 2 and 3 resulted in complete abolishment of the antimicrobial activity. Introduction of a cyanomethyl moiety as in compound **5**, did not offer any advantage over the parent compound **1**, on the contrary, it resulted in a dramatic reduction in the activity against all the tested organisms. On the other hand, introducing a 4-substituted phenylcarbonylmethyl fragment at position 2 resulted in two active compounds (**6**: R = H and **7**: R = Cl). Both were proved to be equipotent to ampicillin against *B. subtilis*, which was even better than the parent thione **1**. It is worth mentioning that replacement of the chlorine atom with bromine one compound, **8**, experienced a total loss of activity. Furthermore, replacement of the aryl group in compounds **6**–**8** with an aliphatic one as in the analog **9**, almost maintained the activity which was about 50% that of ampicillin against *S. aureus, B. Subtilis*, and *P. aeruginosa,* in addition to a moderate antifungal activity against *C. albicans*.

Regarding the thioether derivatives presented in Scheme 2, the key intermediate ester derivative **10** exhibited appreciable activity against all the tested microorganisms with MIC range: 25–50 μg/mL. Conversion of the ester group to an acid hydrazide functionality (compound **11**) did not result in a significant change in the antimicrobial profile, whereas synthesis of the azomethine **13** led to an obvious reduction in both antimicrobial spectrum and potency. Furthermore, replacement of the thioether moiety with a hydrazine counterpart as in **13**, led to a total loss of activity.

Concerning the antimicrobial activity of the second series (Scheme 3), replacement of the thioether moiety with a substituted amino group gave rise to three compounds **14**–**16**, among which the benzyl derivative **14** (R = CH_2-C_6H_5) was the sole analog with moderate activity against Gram-positive bacteria (MIC = 25–100 μg/mL). Chlorination of the benzylamino derivative **14** led to the formation of an obviously active chloropyrimidine analog **17**, with moderate activity against Gram-positive bacteria but with a specially high activity against *B. subtilis* where it exerted the same level of activity as ampicillin. Conversion of the chloro substituent to a secondary amino moiety as in **18** and **19**, resulted in a noticeable reduction in activity. Only one analog namely, **19** (R = C_6H_5), retained the activity against *S. aureus.* It is worth mentioning that, although addition of substituted phenylhydrazines led unexpectedly to cyclized pyrazolopyrimidines **20** and **21**, the latter analog showed considerable antimicrobial activity. Among these, analog **21** (R = SO_2NH_2) exhibited better activity against both Gram-positive and -negative bacteria that was comparable to or even better than that of the parent chloro analog **17**.

On the contrary, introduction of a phenylacetohydrazide moiety as in **22** resulted in a dramatic reduction in activity, whereas the introduction of thiosemicarbazido moiety as in **23** led to a total loss of antimicrobial potential. Finally, increasing compound's lipophilicity through the incorporation of a phenyl thioether counterpart, led to a remarkable change in both the antimicrobial potential and spectrum. When compared with the parent chloroderivative **17**, the analog **24** showed about two-fold reduction in the overall activity against the three tested Gram-positive strains, whereas it exhibited a better anti-Gram-negative potential especially towards *E. coli* (50% of the activity of ampicillin).

Preliminary *in-vitro* anticancer screening

In-vitro MTT cytotoxicity assay

Out of the newly synthesized compounds, eleven analogs namely, **1**, **3**, **5**, **7**, **12**, **13**, **15**, **19**, **21**, **22**, and **24** were selected to be evaluated for their *in-vitro* cytotoxic effect via the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method [45–47], against a panel of three human tumor cell lines. Caucasian breast adenocarcinoma MCF7, hepatocellular carcinoma HePG2, and colon carcinoma HT29. The results are presented in Table 2 as LC_{50} and LC_{90} (μg/mL) which are the lethal concentrations of the compound which cause death of 50% or 90% of the cells in 24 h, respectively.

The obtained data revealed that the three tested human tumor cell lines exhibited variable degrees of sensitivity profiles towards six of the tested compounds namely; **1**, **3**, **7**, **12**, **13**, and **15**, at both the LC_{50} and LC_{90} levels. Among these, the human colon carcinoma HT29 cell line showed pronounced sensitivity towards compound **15** with LC_{50} value of 3.4 μg/mL. Such high activity

Table 2. Cytotoxic effects (LC_{50} and LC_{90}, in μg/mL) of the active compounds on some human tumor cell lines using the MTT assay.

Compound	Human colon carcinoma HT29		Human hepatocellular carcinoma HePG2		Human breast cancer MCF7	
	LC_{50} [a)]	LC_{90} [b)]	LC_{50}	LC_{90}	LC_{50}	LC_{90}
1	41.4	65.2	16.9	28.3	46.4	101
3	55.9	89.7	14.3	37.3	– [c)]	–
7	10.1	34.6	20.9	40.6	1.9	30.4
12	19.4	67.7	26.7	79.4	48.7	93.8
13	21.2	91.3	17.7	47.6	–	–
15	3.4	24.9	19.1	71.5	63.4	156

[a)] LC_{50}: lethal concentration of the compound which causes death of 50% of cells in 24 h (μg/mL).
[b)] LC_{90}: lethal concentration of the compound which causes death of 90% of cells in 24 h (μg/mL).
[c)] Totally inactive against this cell line.

was maintained at the LC_{90} level which showed also the greatest activity (24.9 μg/mL). Moreover, a remarkable cytotoxic potential was displayed by compound **7** against the same cell line at both the LC_{50} and LC_{90} levels (10.1 and 34.6 μg/mL, respectively). Meanwhile, compounds **12** and **13** revealed an obvious cytotoxicity profile against colon carcinoma HT29 at the LC_{50} level (19.4 and 21.2 μg/mL, respectively). Whereas, mild activity was displayed by compounds **1** and **3** at the LC_{50} level (41.4 and 55.9 μg/mL, respectively). Furthermore, the growth of the human hepatocellular carcinoma HePG2 cell line was found to be inhibited by all the six active compounds. It showed nearly the same sensitivity profile towards the six active compounds with LC_{50} values range of 14.3 – 26.7 μg/mL. Among these, the highest cytotoxic activity was displayed by compounds **1** and **3** which were almost equipotent (LC_{50} = 16.9 and 14.3 μg/mL, respectively). However, their LC_{90} pattern was reversed (LC_{90} = 28.3 and 37.3 μg/mL, respectively). On the other hand, human breast cancer MCF7 was proved to be the least sensitive among the cell lines tested as it was affected by only four test compounds. However, an outstanding growth inhibition potential was shown by compound **7** as evidenced from its LC_{50} and LC_{90} values (1.9 and 30.4 μg/mL, respectively). The remaining three active compounds, namely **1**, **12**, and **15** showed mild activity against the same cell line with LC_{50} values of 46.4, 63.4, and 48.7 μg/mL, respectively.

Further interpretation of the results revealed that compounds **7** and **15** showed a considerable broad spectrum of cytotoxic activity against the three tested human tumor cell lines at both the LC_{50} and LC_{90} levels. In particular, compound **7** proved to be the most active member in this study with a broad spectrum of activity against the tested cell lines, with special effectiveness against the human colon carcinoma HT29 and human breast cancer MCF7 cell lines at both the LC_{50} and LC_{90} levels (LC_{50} = 10.1, 1.9 and LC_{90} = 34.6, 30.4 μg/mL, respectively).

Conclusion

In conclusion, the present study focussed on the synthesis and investigation of the antimicrobial and cytotoxic activity of two series of polysubstituted pyrimidines with the hope of discovering new structure leads serving as potential broad spectrum antimicrobial and / or anticancer agents. The obtained results revealed that 18 compounds were able to display variable growth inhibitory effects on the tested Gram-positive and Gram-negative bacterial strains, among them, six compounds exhibited some moderate antifungal activity against *C. albicans*. Globally, most of the tested compounds showed better activity against the Gram-positive rather than the Gram-negative bacteria, particularly *S. aureus* and *B. subtilis*. Structurally, compounds belonging to the first series (thioethers) displayed better overall antimicrobial activity than that of the second one (substituted amines). Among these, compounds **1**, **2**, **6**, **7**, **9**, **10**, **11**, **21**, and **24** revealed a considerable broad spectrum of antibacterial activity against both Gram-positive and negative bacteria. Moreover, compounds **1**, **2**, **6**, **7**, **9**, and **24** exhibited an appreciable antifungal activity against *C. albicans* comparable to that of clotrimazole. An outstanding broad spectrum antibacterial profile was displayed by compound **2** which was proved to be twice as active as ampicillin against *B. subtilis* and equipotent to ampicillin against *M. Luteus,* and *P. aeruginosa,* together with a moderate antifungal activity. Consequently, it is considered to be the most active antimicrobial member identified in this study.

On the other hand, eleven analogs were evaluated for their *in-vitro* cytotoxic effect utilizing the standard MTT assay, against a panel of three human cell lines: breast adenocarcinoma MCF7, hepatocellular carcinoma HePG2, and colon carcinoma HT29. The obtained data revealed that six of the tested compounds namely; **1**, **3**, **7**, **12**, **13**, and **15** showed a variable degree of cytotoxic activity against the tested cell lines at both the LC_{50} and LC_{90}

Table 3. Physicochemical and analytical data for compounds **1**–**24**.

Compound	R	Yield (%)	Mp. (°C) Cryst. Solvent[a)]	Molecular formula (Mol. wt.)[b)]
1	–	65	250–252 (D)	$C_{19}H_{15}N_3O_3S$ (365.41)
2	CH_3	32	174–176 (D / E)	$C_{20}H_{17}N_3O_3S$ (379.43)
3	$C_6H_5CH_2$	70	234–35 (D / E)	$C_{26}H_{21}N_3O_3S$ (455.53)
4	–	75	206–207 (D / E)	$C_{21}H_{19}N_3O_3S$ (393.46)
5	–	78	188–190 (D / E)	$C_{21}H_{16}N_4O_3S$ (404.44)
6	H	80	208–210 (D)	$C_{27}H_{21}N_3O_4S$ (483.55)
7	Cl	80	220–222 (DMF / E)	$C_{27}H_{20}ClN_3O_4S$ (517.98)
8	Br	82	> 300 (DMF / E)	$C_{27}H_{20}BrN_3O_2$ (562.44)
9	–	75	178–179 (EA)	$C_{22}H_{19}N_3O_4S$ (421.47)
10	–	81	223–224 (D)	$C_{23}H_{21}N_3O_5S$ (451.50)
11	–	60	292–293 (E)	$C_{21}H_{19}N_5O_4S$ (437.47)
12	–	82	246–247 (DMF / W)	$C_{19}H_{17}N_5O_3$ (363.37)
13	–	68	294–296 (D)	$C_{28}H_{22}ClN_5O_4S$ (560.02)
14	$C_6H_5CH_2$	75	246–248 (E)	$C_{26}H_{22}N_4O_3$ (438.48)
15	C_6H_5	67	218–219 (D)	$C_{25}H_{20}N_4O_3$ (424.45)
16	cyclo-C_6H_{11}	31	213–214 (E)	$C_{25}H_{26}N_4O_3 \cdot 1.5\,H_2O$ (457.53)
17	–	52	133–135 (E / PE.)	$C_{26}H_{21}ClN_4O_2$ (456.92)
18	$C_6H_5CH_2$	76	144–145 (D / E)	$C_{33}H_{29}N_5O_2 \cdot 0.5\,H_2O$ (536.64)
19	C_6H_5	76	122–124 (E)	$C_{32}H_{27}N_5O_2 \cdot 0.5\,H_2O$ (522.61)
20	H	67	216–217 (E)	$C_{32}H_{28}N_6O_2$ (528.60)
21	SO_2NH_2	81	210–211 (DMF / W)	$C_{32}H_{29}N_7O_4S$ (607.68)
22	–	52	168–169 (E)	$C_{34}H_{30}N_6O_3$ (570.64)
23	–	53	230–231 (D)	$C_{27}H_{25}N_7O_2S \cdot H_2O$ (529.62)
24	–	70	170–171 (D / E)	$C_{32}H_{26}N_4O_2S \cdot H_2O$ (548.67)

[a)] Crystallization solvent(s): D: dioxane, DMF: *N*,*N*-dimethylformamide, E: ethanol, EA: ethyl acetate, PE.: petroleum ether (60 : 80), W: water.

[b)] Found values are within ± 0.4% of the calculated values.

levels. Among these, compounds **7** and **15** showed considerable broad spectrum of cytotoxic activity against the three tested human tumor cell lines. Compound **7** was proved to be the most active member in this study with a broad spectrum of activity against the tested cell lines, with special effectiveness against the human colon carcinoma HT29 and human breast cancer MCF7 cell lines at both the LC_{50} and LC_{90} levels. Collectively, the antimicrobial and cytotoxic results suggest that compounds **1** and **7** could be considered as possible dual antimicrobial-anticancer candidates that deserve further investigation and derivatization in order to explore the scope and limitation of their biological activities. Physiochemical and analytical data are recorded in Table 3.

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The authors have declared no conflict of interest.

Experimental

Chemistry

Melting points were determined in open glass capillaries using Stuart Capillary melting point apparatus (Stuart Scientific Stone, Staffordshire, UK) and are uncorrected. Infrared (IR) spectra were recorded on Perkin-Elmer 1430 infrared spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). ^{1}H-NMR spectra were scanned on Jeol-500 MHz spectrometer (Jeol, Japan) using tetramethylsilane (TMS) as internal standard and DMSO-d_6 as the solvent (chemical shifts are given in δ ppm). Splitting patterns were designated as follows; s: singlet; br s: broad singlet d: doublet; dd: double doublet; t: triplet; m: multiplet. Mass spectra were carried out using a Schimadzu GCMS-QP-1000EX mass spectrometer (Shimadzu, Tokyo, Japan) at 70 ev, Faculty of Science, Cairo University. Elemental analyses were performed at the microanalytical unit, Faculty of Science, Cairo University and were within ± 0.4% of the theoretical values. Follow-up of the reactions and checking the purity of the compounds was made by thin layer chromatography (TLC) on silica gel-precoated aluminium sheets (Type 60 GF_{254}; Merck, Germany) and the spots were detected by exposure to UV lamp at λ_{254} for a few seconds.

6-(4-Benzyloxy-3-methoxyphenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile ***1***

A mixture of thiourea (1.9 g, 25 mmol), ethyl cyanoacetate (2.83 g, 2.66 mL, 25 mmol), 4-benzyloxy-3-methoxybenzaldehyde (6 g, 25 mmol), and anhydrous K_2CO_3 (3.45 g, 25 mmol) in ethanol (150 mL) was heated under reflux for 12 h. After being

cooled to room temperature, the obtained precipitate was filtered and washed with ethanol. The product was then dissolved in hot water, filtered while hot and neutralized with conc. HCl whereupon the desired thione was obtained. It was filtered, washed with water, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3311, 3212 (NH), 2234 (CN), 1694 (C=O), 1530, 1306, 1153, 1048 (N-C=S), 1235, 1025 (C-O-C).^{1}H-NMR δ (ppm): 3.78 (s, 3H, OCH_3), 5.15 (s, 2H, OCH_2), 7.17 (d, *J* = 8.6 Hz, 2H, alkoxyphenyl C_5-H), 7.27-7.47 (m, 7H, benzyloxy-H & alkoxyphenyl $C_{2,6}$-H), 9.8, 12.77 (2s, 2H, 2NH, D_2O exchangeable).

*4-(4-Benzyloxy-3-methoxyphenyl)-2-(methyl or benzyl)sulfanyl-6-oxo-1,6-dihydro-pyrimidine-5-carbonitriles **2, 3***

To a suspension of **1** (0.7 g, 1.9 mmol) in DMF (5 mL) containing anhydrous K_2CO_3 (0.26 g, 1.9 mmol), methyl iodide (compound **2**) or benzyl chloride (compound **3**) (1.9 mmol) was added. The reaction mixture was stirred at room temperature for 30 min (for **2**) or for overnight (for **3**), then poured onto cold water and the formed precipitate was filtered, washed with water, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3196 – 3148 (NH), 2214 – 2204 (CN), 1649 – 1648 (C=O), 1598 – 1595 (C=N).^{1}H-NMR δ (ppm) for **3**: 3.73 (s, 3H, OCH_3), 4.52 (s, 2H, SCH_2), 5.16 (s, 2H, OCH_2), 7.19 – 7.45 (m, 11H, alkoxyphenyl C_5-H, benzylsulfanyl-H & benzyloxy-H), 7.57 (s, 1H, alkoxyphenyl C_2-H), 7.61 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_6-H).

*4-(4-Benzyloxy-3-methoxyphenyl)-1-methyl-2-methylsulfanyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **4***

To a solution of equimolar amounts of the thione **1** (0.7 g, 1.9 mmol) and anhydrous K_2CO_3 (0.26 g, 1.9 mmol) in dry DMF (5 mL), methyl iodide (0.82 g, 0.36 mL, 5.7 mmol) was added and the reaction mixture was stirred at room temperature overnight. The mixture was poured onto crushed ice and the obtained solid product was filtered, washed with water, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 2214 (CN), 1665 (C=O), 1593 (C=N). ^{1}H-NMR δ(ppm): 2.62 (s, 3H, N-CH_3), 3.39 (s, 3H, SCH_3), 3.79 (s, 3H, OCH_3), 5.13 (s, 2H, OCH_2), 7.20 (d, *J* = 8.98 Hz, 1H, alkoxyphenyl C_5-H), 7.32 (t, *J* = 7.65 Hz, 1H, benzyloxy C_4-H), 7.38 (t, *J* = 7.65 Hz, 2H, benzyloxy $C_{3,5}$-H), 7.43 (d, *J* = 7.65 Hz, 2H, benzyloxy $C_{2,6}$-H), 7.65 (s, 1H, alkoxyphenyl C_2-H), 7.67 (dd, *J* = 8.98, 2.68 Hz, 1H, alkoxyphenyl C_6-H).

*4-(4-Benzyloxy-3-methoxyphenyl)-2-cyanomethylsulfanyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **5***

To a suspension of **1** (0.37 g, 1 mmol) in dry DMF (3 mL) containing anhydrous K_2CO_3 (0.14 g, 1 mmol), chloroacetonitrile (0.08 g, 0.06 mL, 1 mmol) was added. The reaction mixture was stirred at room temperature overnight, poured onto crushed ice, then acidified with dilute HCl. The obtained precipitate was filtered, washed with H_2O, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3144 (NH), 2219 (CN), 1650 (C=O), 1615 (C=N). ^{1}H-NMR δ (ppm): 3.75 (s, 3H, OCH_3), 4.12 (s, 2H, SCH_2), 5.13 (s, 2H, OCH_2), 7.20 (d, *J* = 8.98 Hz, 1H, alkoxyphenyl C_5-H), 7.30 – 7.40 (m, 5H, benzyloxy-H), 7.66 (s, 1H, alkoxyphenyl C_2-H), 7.67 (dd, *J* = 8.98, 2.68 Hz, 1H, alkoxyphenyl C_6-H).

*General procedure for the synthesis of 4-(4-benzyloxy-3-methoxyphenyl)-6-oxo-2-(substituted phenylcarbonylmethyl- or 2-oxoprop-1-ylsulfanyl)-1,6-dihydropyrimidine-5-carbonitriles **6 – 9***

A mixture of **1** (0.37 g, 1 mmol), the appropriate phenacyl bromide (for **6 – 8**) (1 mmol) or, chloroacetone (for **9**) (0.09 g, 0.08 mL, 1 mmol), and anhydrous K_2CO_3 (0.14 g, 1 mmol) in dry DMF (3 mL) was stirred at room temperature overnight. Working up of the reaction mixture was carried out as described under compound **5**. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3415 – 3109 (NH), 2214 – 2212 (CN), 1692 – 1650 (C=O), 1612 – 1585 (C=N). ^{1}H-NMR δ (ppm) for **7** (R = Cl): 3.61 (s, 3H, OCH_3), 4.87 (s, 2H, SCH_2), 5.08 (s, 2H, OCH_2), 6.92 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.26 – 7.43 (m, 7H, benzyloxy-H and alkoxyphenyl $C_{2,6}$-H), 7.55, 7.99 (2d, *J* = 8.4 Hz, 4H, chlorophenyl $C_{3,5}$-H and $C_{2,6}$-H). ^{1}H-NMR δ (ppm) for **9**: 2.61 (s, 3H, O=C-CH_3), 3.03 (s, 2H, SCH_2), 3.81 (s, 3H, OCH_3), 5.17 (s, 2H, OCH_2), 7.23 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.33 (t, *J* = 7.65 Hz, 1H, benzyloxy C_4-H), 7.39 (t, *J* = 7.65 Hz, 2H, benzyloxy $C_{3,5}$-H), 7.45 (d, *J* = 7.65 Hz, 2H, benzyloxy $C_{2,6}$-H), 7.57 (s, 1H, alkoxyphenyl C_2-H), 7.62, 7.64 (dd, *J* = 8.4, 2.3 Hz, 1H, alkoxyphenyl C_6-H).

*Ethyl 2-[4-(4-Benzyloxy-3-methoxyphenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl-sulfanyl]acetate **10***

To a suspension of **1** (0.37 g, 1 mmol) in dry DMF (3 mL) containing anhydrous K_2CO_3 (0.14 g, 1 mmol), ethyl chloroacetate (0.12 g, 1 mmol) was added. The reaction mixture was stirred at room temperature for 6 h, poured onto crushed ice. The obtained precipitate was filtered, washed with H_2O, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3296 (NH), 2218 (CN), 1736, 1654 (C=O), 1596 (C=N).^{1}H-NMR δ (ppm): 1.01 (t, *J* = 6.9 Hz, 3H, CH_2CH_3), 3.82 (s, 3H, OCH_3), 3.99 (q, *J* = 6.9 Hz, 2H, CH_2CH_3), 4.12 (s, 2H, SCH_2), 5.18 (s, 2H, OCH_2), 7.18 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.32 (t, *J* = 7.02 Hz, 1H, benzyloxy C_4-H), 7.37 (t, *J* = 7.02 Hz, 2H, benzyloxy $C_{3,5}$-H), 7.43 (d, *J* = 7.02 Hz, 2H, benzyloxy $C_{2,6}$-H), 7.53 – 7.58 (m, 2H, alkoxyphenyl $C_{2,6}$-H), 12.4 (s, 1H, NH, D_2O exchangeable).

*2-[4-(4-Benzyloxy-3-methoxyphenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-ylsulfanyl]acetic acid hydrazide **11***

To a suspension of **10** (1 g, 2.2 mmol) in absolute ethanol (10 mL) hydrazine hydrate 98% (0.55 g, 0.53 mL, 11 mmol) was added and the mixture was stirred at room temperature for 3 h. The reaction mixture cleared after 1 h, then a white precipitate separated out. After 3 h, the precipitate was filtered, washed with ethanol, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3347, 3279, 3186 (NH_2, NH), 2205 (C≡N), 1647 (C=O), 1604 (C=N). ^{1}H-NMR δ (ppm): 3.71 (s, 3H, OCH_3), 3.78 (s, 2H, NH_2, D_2O exchangeable), 4.80 (s, 2H, SCH_2), 5.16 (s, 2H, OCH_2), 7.12 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.30-7.60 (m, 7H, benzyloxy-H & alkoxyphenyl $C_{2,6}$-H), 9.4, 12.4 (2s, 2H, 2NH, D_2O exchangeable).

*4-(4-Benzyloxy-3-methoxyphenyl)-2-hydrazino-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **12***

A mixture of **10** (1 g, 2.2 mmol) and hydrazine hydrate 98% (0.22 g, 0.21 mL, 4.4 mmol) in absolute ethanol (20 mL) was heated under reflux for 2 h during which a crystalline white precipitate separated out. It was filtered, washed with water, dried, and crystallized. Physicochemical and analytical data are

recorded in Table 3. IR KBr (cm^{-1}): 3439, 3275 (NH_2, NH), 2205 (CN), 1667 (C=O), 1591 (C=N).^{1}H-NMR δ (ppm): 3.43 (br s, 2H, NH_2, D_2O exchangeable), 3.77 (s, 3H, OCH_3), 5.13 (s, 2H, OCH_2), 7.12 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.31 (t, *J* = 7.46 Hz, 1H, benzyloxy C_4-H), 7.37 (t, *J* = 7.46 Hz, 2H, benzyloxy $C_{3,5}$-H), 7.41–7.50 (m, 4H, benzyloxy $C_{2,6}$-H & alkoxyphenyl $C_{2,6}$-H), 9.40, 12.5 (2s, 2H, 2NH, D_2O exchangeable).

N^1-(4-chlorobenzylidene)-2-[4-(4-benzyloxy-3-methoxyphenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-ylsulfanyl]acetic acid hydrazide ***13***

A suspension of the acid hydrazide **11** (0.6 g, 1.37 mmol) in ethanol (10 mL) was heated under reflux with an equimolar amount of 4-chlorobenzaldehyde (0.19 g, 1.37 mmol) for 2 h. The reaction mixture was left to attain room temperature and the separated solid product was filtered, washed with cold ethanol, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3155 (NH), 2209 (CN), 1652 (C=O), 1600 & 1585 (C=N). ^{1}H-NMR δ (ppm): 3.51 (s, 2H, SCH_2), 3.79 (s, 3H, OCH_3), 5.11 (s, 2H, OCH_2), 7.19 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.33 (t, *J* = 7.65 Hz, 1H, benzyloxy C_4-H), 7.38 (t, *J* = 7.65 Hz, 2H, benzyloxy $C_{3,5}$-H), 7.44 (d, *J* = 7.65 Hz, 2H, benzyloxy $C_{2,6}$-H), 7.47 (d, *J* = 8.8 Hz, 2H, chlorophenyl $C_{3,5}$-H), 7.49 (s, 1H, alkoxyphenyl C_2-H), 7.51 (dd, *J* = 8.4, 2.3 Hz, 1H, alkoxyphenyl C_6-H), 8.05 (d, *J* = 8.8 Hz, 2H, chlorophenyl $C_{2,6}$-H), 8.13 (s, 1H, N=CH), 12.5 (br s, 2H, 2NH, D_2O exchangeable).

4-(4-Benzyloxy-3-methoxyphenyl)-2-substituted amino-6-oxo-1,6-dihydropyrimidine-5-carbo-nitriles ***14–16***

A mixture of **10** (1 g, 2.2 mmol) and the selected amine (8.8 mmol) was fused in an oil bath at 160–170°C for 30 min. The reaction mixture was left to cool and then triturated with diethyl ether (2 × 15 mL). The obtained precipitate was filtered, washed with ether, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR (KBr, cm^{-1}): 3395–3211 (NH), 2212–2210 (CN), 1665–1652 (C=O), 1616–1613 (C=N). ^{1}H-NMR δ(ppm) for **14** (R = $CH_2C_6H_5$): 3.7 (s, 3H, OCH_3), 4.58 (d, *J* = 5.35 Hz, 2H, NH-CH_2), 5.13 (s, 2H, OCH_2), 7.13 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_5-H), 7.22 (t, *J* = 7.25 Hz, 1H, benzylamino C_4-H), 7.28–7.35 (m, 5H, benzylamino $C_{2,3,5,6}$-H & benzyloxy C_4-H), 7.36–7.40 (m, 3H, benzyloxy $C_{3,5}$-H and alkoxyphenyl C_2-H), 7.43 (d, *J* = 6.9 Hz, 2H, benzyloxy $C_{2,6}$-H), 7.48 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_6-H), 7.87, 11.82 (2s, 2H, 2NH, D_2O exchangeable).

2-Benzylamino-4-(4-benzyloxy-3-methoxyphenyl)-6-chloropyrimidine-5-carbonitrile ***17***

A solution of **14** (5 g, 11.4 mmol) in $POCl_3$ (20 mL) was heated under reflux for 15 min. The reaction mixture was left to attain room temperature, then poured onto crushed ice. The precipitated product was filtered, washed with water, dried, and purified by column chromatography using CH_2Cl_2 / petroleum ether 60/80 (120 : 10) as eluting solvent. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3240 (NH), 2198 (CN), 1597 (C=N). ^{1}H-NMR δ (ppm): 3.45, 3.69 (2d, *J* = 18 Hz, 2H, NHC*H*$_2$), 4.03 (s, 3H, OCH_3), 5.08 (s, 2H, OCH_2), 7.01–7.40 (m, 13H, Ar-H), 7.8 (s, 1H, NH, D_2O exchangeable).

2-Benzylamino-4-(4-benzyloxy-3-methoxyphenyl)-6-substituted aminopyrimidine-5-carbonitriles ***18, 19***

A suspension of **17** (0.5 g, 1.1 mmol) and the appropriate amine (2.2 mmol) in ethanol (15 mL) was heated under reflux for 6 h. The reaction mixture was left overnight whereupon a crystalline white solid separated out. It was filtered, washed with ether, dried, and recrystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3365–3242 (NH), 2198–2197 (CN), 1604–1590 (C=N).^{1}H-NMR δ (ppm) for **18**: 3.77 (s, 3H, OCH_3), 4.41 (d, *J* = 6.1 Hz, 2H, pyrimidine-C_2-NHC*H*$_2$), 4.53 (d, *J* = 5.9 Hz, 2H, pyrimidine-C_4-NHC*H*$_2$), 5.12 (s, 2H, OCH_2), 7.09–7.46 (m, 18H, Ar-H), 7.99 (t, *J* = 6.1 Hz, 1H, pyrimidine-C_2-NH), 8.23 (t, *J* = 5.9 Hz, 1H, pyrimidine-C_4-NH).

3-Amino-6-benzylamino-4-(4-benzyloxy-3-methoxyphenyl)-1-phenylpyrazolo[3,4-d]pyrimidine ***20*** *and 4-[3-amino-6-benzylamino-4-(4-benzyloxy-3-methoxyphenyl)pyrazolo[3,4-d]pyrimidin-1-yl]benzenesulfonamide* ***21***

A suspension of **17** (0.5 g, 1.1 mmol), the appropriate aryl hydrazine hydrochloride (1.1 mmol) and anhydrous sodium acetate (0.09 g, 1.1 mmol) in absolute ethanol (15 mL) was heated under reflux for 12 h. The reaction mixture was left overnight and the separated solid product was filtered, washed with ethanol, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3398–3350, 3289–3256 & 3155–3125 (NH_2 & NH), 1636–1589 (C=N).^{1}H-NMR δ (ppm) for **20**: 3.60 (br s, 2H, NH_2, D_2O exchangeable), 3.83 (s, 3H, OCH_3), 4.58 (s, 2H, $NHCH_2$), 5.16 (s, 2H, OCH_2), 7.15–7.58 (m, 17H, Ar-H), 7.98 (d, *J* = 8.4 Hz, 1H, alkoxyphenyl C_6-H), 8.53 (br s, 1H, NH, D_2O exchangeable). MS m/z (relative abundance%) for compound **20**: 529 [M^+ + 1] (4.5), 528 [M^+] (7.6), 438 (6.4), 437 (4.7), 91 (100), 90 (4.7), 89 (2.9), 78 (2.5), 77 (10.7), 66 (3.7), 65 (11.9).

General procedure for the synthesis of 2-benzylamino-4-(4-benzyloxy-3-methoxyphenyl)-6-substituted pyrimidine-5-carbonitriles ***22, 23***

A mixture of **17** (0.5 g, 1.1 mmol) and phenyl acetic acid hydrazide (for **22**) (0.17 g, 1.1 mmol); or thiosemicarbazide (for **23**) (0.12 g, 1.3 mmol) in ethanol (20 mL) was heated under reflux for 18 h. The reaction mixture was left overnight and the precipitated product was filtered, washed with ethanol, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3436–3418 & 3335–3283 (NH_2 & NH), 2211–2205 (CN), 1669 (C=O), 1630–1603 (C=N). ^{1}H-NMR δ(ppm) for **22**: 3.77 (s, 3H, OCH_3), 4.35 (s, 2H, O=C-CH_2), 4.52 (d, *J* = 6.5 Hz, 2H, NHC*H*$_2$), 5.12 (s, 2H, OCH_2), 7.11–7.45 (m, 18H, Ar-H), 8.31 (t, *J* = 6.5 Hz, 1H, N*H*CH_2, D_2O exchangeable), 9.25, 10.15 (2s, 2H, 2NH, D_2O exchangeable). ^{1}H-NMR δ(ppm) for **23**: 3.54 (s, 2H, NH_2, D_2O exchangeable), 3.83 (s, 3H, OCH_3), 4.61 (s, 2H, NHC*H*$_2$), 5.17 (s, 2H, OCH_2), 7.19–7.50 (m, 13H, Ar-H), 8.09, 8.48, 12.96 (3s, 3H, 3NH, D_2O exchangeable).

2-Benzylamino-4-(4-benzyloxy-3-methoxyphenyl)-6-phenylsulfanylpyrimidine-5-carbonitrile ***24***

To a well stirred suspension of **17** (0.5 g, 1.1 mmol) and anhydrous K_2CO_3 (0.15 g, 1.1 mmol) in dry DMF (5 mL), an equimolar amount of thiophenol (0.12 g, 0.11 mL) was added and the mixture was left stirred at room temperature overnight. The reac-

tion mixture was poured onto crushed ice and the precipitated product was filtered, washed with water, dried, and crystallized. Physicochemical and analytical data are recorded in Table 3. IR KBr (cm^{-1}): 3403 (NH), 2206 (CN), 1595 (C=N). ^{1}H-NMR δ (ppm): 3.78 (s, 3H, OCH_3), 4.06 (d, J = 6.47 Hz, 2H, $NHCH_2$), 5.14 (s, 2H, OCH_2), 6.76 – 7.62 (m, 18H, Ar-H), 8.83 (t, J = 6.47 Hz, 1H, NH, D_2O exchangeable).

Biology

In-vitro antibacterial and antifungal activities

Standard sterilized filter paper discs (5 mm diameter) impregnated with a solution of the test compound in DMSO (1 mg/mL) were placed on an agar plate seeded with the appropriate test organism in triplicates. The utilized test organisms were: *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (NRRL B-14819), and *Micrococcus luteus* (ATCC 21881) as examples of Gram-positive bacteria and *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumonia* (clinical isolate) as examples of Gram-negative bacteria. They were also evaluated for their *in-vitro* antifungal potential against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (recultured) fungal strains were utilized as representatives for fungi. Ampicillin trihydrate and clotrimazole were used as standard antibacterial and antifungal agents, respectively. DMSO alone was used as control at the same above-mentioned concentration. The plates were incubated at 37°C for 24 h for bacteria and for seven days for fungi. Compounds that showed significant growth inhibition zones (≥14 mm), using the two-fold serial dilution technique, were further evaluated for their minimal inhibitory concentrations (MICs).

Minimal inhibitory concentration (MIC) measurement

The microdilution susceptibility test in Müller – Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) was used for the determination of antibacterial and antifungal activity, respectively [44]. Stock solutions of the tested compounds, ampicillin trihydrate and clotrimazole were prepared in DMSO at concentration of 800 μg/mL followed by two-fold dilution at concentrations of (400, 200, …, 6.25 μg/mL). The microorganism suspensions at 10^6 CFU/mL (Colony Forming Unit/mL) concentration were inoculated to the corresponding wells. Plates were incubated at 36°C for 24 to 48 h and the minimal inhibitory concentrations (MIC) were determined. Control experiments were also done.

In-vitro MTT cytotoxicity assay

All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (SG403INT, Baker, Stanford, ME, USA). The method was carried out according to Thabrew *et al*. [47]. Cells were batch-cultured for ten days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of the test compounds to give a final concentration of (100, 50, 25, 12.5, 3.125, 1.56, and 0.78 μg/mL). DMSO was employed as a vehicle for dissolution of the tested compounds and its final concentration on the cells was less than 0.2%. Cells were suspended in RPMI 1640 medium (for HePG2 and HT29 cell lines) and DMEM (for MCF7 cell line), 1% antibiotic-antimycotic mixture (10 000 IU/mL penicillin potassium, 10 000 μg/mL streptomycin sulphate, and 25 μg/mL amphotericin B), and 1% L-glutamine in 96-well flat bottom microplate at 37°C under 5% CO_2. After 48 h of incubation, the medium was aspirated, 40 μL of MTT salt (2.5 μg/mL) were added to each well and incubated for further 4 h at 37°C under 5% CO_2. To stop the reaction and dissolve the formed crystals, 200 μL of 10% sodium dodecyl sulphate (SDS) in de-ionized water was added to each well and incubated overnight at 37°C. A positive control which composed of 100 μg/mL of Annona cherimolia extract was used as known cytotoxic natural agent that gives 100% lethality under the same experimental conditions. The absorbance was then measured using a microplate multi-well reader (model 3350, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 595 nm and a reference wavelength of 620 nm.

Statistical significance was tested between samples and negative control (cells with vehicle) using independent *t*-test by SPSS 11 program. The percentage change in viability was calculated according to the formula:

[Reading of the sample/

$$(\text{Reading of the negative control} - 1)] \times 100 \qquad (1)$$

A probit analysis was carried for LC_{50} and LC_{90} determinations using SPSS 11 program.

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