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Synthesis, cytotoxicity and in vitro antibacterial screening of novel hydrazones bearing thienopyridine moiety as potent COX-2 inhibitors

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Abstract

The starting precursors 2-carbohydrazides, bearing thienopyridine moiety, were prepared using 2-thioxopyridine-3-carbonitriles as key synthons. Next, 2-carbohydrazides were reacted with a variety of 4-substituted benzylidinemalononitriles or 4-substituted benzaldehydes to afford a new series of the target hydrazones incorporating thienopyridine moiety. The elemental analyses and spectral data were used to demonstrate the structures of new hydrazones series. The in vitro antibacterial activities of the target hydrazones were evaluated against different strains of Gram-positive and Gram-negative bacteria. In comparison with chloramphenicol as a reference drug, hydrazones **13b**, **13c** and **13d**, linked to *p*-Cl, *p*-Br and *p*-Me moiety, respectively, exhibited the strongest activities against all tested bacteria with MIC values in the range of $6.2-12.5 \mu g/mL$. In addition, several new hydrazones were tested as in vitro cytotoxic agents against each of human breast carcinoma MCF-7 cell line, colon cancer Caco2 cell line and liver hepatocellular carcinoma HEPG2 cell line. The hydrazones **13b**, **13c** and **13d** demonstrated the best cytotoxicity against the tested eukaryotic cells. Furthermore, both experimental and docking studies could predict the promising inhibitory activities of hydrazones **13c** and **13d** against COX-2 enzyme with IC₅₀ of 0.110 and 0.104 μ M, respectively, when compared with Celecoxib (IC₅₀ of 0.115 μ M).

Graphic abstract



Keywords Hydrazones \cdot Thieno[2,3-b]pyridine \cdot In vitro antibacterial screening \cdot COX-2 inhibitors \cdot In silico study

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Introduction

In recent years, design of synthetic routes for preparation of new thieno[2,3-b]pyridines has attracted considerable attention because of their important medicinal activities as antimicrobial [1, 2], antiviral [3, 4] and anti-inflammatory agents [5–7]. Also, these derivatives exhibit interesting antiproliferative and anticancer activities [8–11] as well as their capability for treatment of disorders of central nervous system [12–14]. Moreover, thienopyridines

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exhibit broad spectrum of bioactivities as antidiabetic [15, 16], antihypertensive [17, 18] and osteogenic [19, 20] activities.

On the other hand, many publications reported the remarkable biological and pharmacological properties of several hydrazone containing derivatives as antituberculosis [21], antibacterial, antifungal [22–24], anticonvulsant [25], antimalarial [26] and anti-HIV [27] agents.

In connection with our efforts in designing facile procedures for the preparation of thienopyridines and their related fused heterocyclic derivatives [28–36], the goal of this work was aimed to construct an efficient procedure for the synthesis of a new series of hydrazones incorporating thienopyridine moieties. Evaluation of the in vitro antimicrobial potencies of the target hydrazones was performed against various Gram-positive and Gramnegative bacteria. The in vitro cytotoxicity of some new hydrazones was evaluated against several eukaryotic cell lines. In addition, in vitro and in silico studies were performed to predict the capability of new hydrazones as potent COX-2 enzyme inhibitors using Celecoxib as a reference drug.

Results and discussion

Chemistry

Initially, carbonyl derivatives **3a,b** were prepared by the reaction of thiophene-2-carboxaldehyde **1** with 4-substituted acetophenones **2a,b** [37]. Compounds **3a,b** were reacted with 2-cyanothoacetamide **4** in ethanol in the presence of piperidine at reflux to give the starting precursors 2-thiox-opyridine-3-carbonitriles **5a,b** (see Scheme 1 and "Experimental" section) [38].

Then, 2-thioxopyridine-3-carbonitriles **5a,b** were reacted with ethyl 2-chloroacetate **6** in ethanol in the presence of sodium acetate under constant stirring at room temperature to give ethyl 2-((pyridin-2-yl)thio)acetates **7a,b**. The reaction most probably proceeded via a simple nucleophilic substitution reaction (Scheme 2). Both elemental analyses and spectral data were performed to confirm the chemical structures of **7a,b**. The IR spectrum of **7a**, as a representative example, showed both nitrile and carbonyl functions at 2214 and 1729 cm⁻¹. Its ¹H-NMR spectrum showed a triplet, a quartet and a singlet signals at δ 1.14, 4.10 and 4.24 corresponding to OCH₂CH₃, OCH₂CH₃ and SCH₂



Scheme 1 Synthesis of 2-thioxopyridine-3-carbonitriles 5



Scheme 2 Synthesis of nicotinonitriles 7 and carbohydrazides 9

protons, respectively. Its ¹³C-NMR spectrum revealed five signals at δ 14.4, 33.1, 61.6, 116.2 and 169.0 corresponding to CH₂CH₃, CH₂CH₃, SCH₂, CN and CO carbons (see Experimental section).

The isolation of nicotinonitriles **7a,b** with ethoxycarbonyl group stimulated our interest to investigate their reaction with nucleophiles. Therefore, the reaction between 7a and hydrazine hydrate in pyridine at reflux was investigated. The IR spectrum of the above product showed the absence of cyano group and the presence of NH and NH₂ groups at 3396, 3344, 3188 cm⁻¹. Its ¹H-NMR spectrum showed the absence of any COOCH₂CH₃ or SCH₂ protons. Instead, it showed three singlet signals at δ 4.51, 6.11 and 9.15 corresponding to NHN H_2 , thiophene-N H_2 and NH protons (see Experimental section). Based on these findings, the product was assigned as 3-aminothieno[2,3-b]pyridine-2-carbohydrazide 9a. Likewise, the reaction of 7b with hydrazine hydrate in pyridine afforded the corresponding 2-carbohydrazide **9b**. The reaction probably proceeded by the initial nucleophilic acyl substitution reaction to give 2-((pyridin-2-yl)thio)acetohydrazide [8] followed by intramolecular cyclization via addition of methylene group to cyano group to give carbohydrazide 9, bearing thienopyridine moiety (Scheme 2).

The structure of 9a was more confirmed via its independent synthesis by another two-step synthetic route. Thus, nicotinonitrile derivative 7a reacted with ethanolic sodium ethoxide solution at reflux to afford ethyl thieno[2,3-*b*] pyridine-2-carboxylate 10 via intramolecular cyclization. Then, 2-carboxylate 10 reacted with hydrazine hydrate to afford 2-carbohydrazide 9a (see Scheme 3 and Experimental section).

Next, carbohydrazides **9a,b** were taken as key synthons to prepare the target hydrazones, incorporating thienopyridine moiety. Therefore, **9a** was reacted with benzylidinemalononitrile **11a** in pyridine at reflux. TLC analysis was used to follow up the reaction. After 4 h of reaction, TLC showed the separation of one product with some slight impurities. After recrystallization, a sole product was isolated which formulated as the corresponding N'-benzylidenethienopyridine-2-carbohydrazide **13a**. The reaction most probably proceeded via an initial hetero-Michael addition of N-NH₂ group to the C=C bond in **11a** to give [12] followed by an intramolecular nucleophilic attack of the NH group with subsequent removal of one molecule of malononitrile (Scheme 4) [30, 39].

In the same way, **9a,b** were reacted with a variety of 4-substituted benzylidinemalononitrile **11b–11k** to give the corresponding hydrazones **13b–13k** and **14a–14f** (Scheme 4). TLC analyses were performed to determine the reaction times. The ¹H-NMR spectrum of **13f**, as a representative example, revealed five singlet signals at δ 3.83, 6.71, 7.86, 8.04 and 11.43 attributed to OCH₃, NH₂, pyridine-H5, methine-H and NH protons (see Experimental section).

It was reported the presence of hydrazones prepared from substituted hydrazides in the *E*-configuration at N=CH bond [40, 41]. So, the configuration of the target hydrazones was assigned as (E)-13(14)A and not the (Z)-isomers 13(14)B (Scheme 4).

The structure of target hydrazones **13** was confirmed chemically via their synthesis by another route. So, hydrazones **13a**, **13b** and **13d** were prepared by the direct condensation of carbohydrazide **9** with the appropriate aromatic aldehydes **15a–15c** in pyridine (Scheme 5).

Biology

Antibacterial screening

The in vitro antibacterial activities were estimated for the starting precursors **7** and **9** as well as the new series of hydrazones **13** and **14**, bearing thienopyridine moiety, using agar well diffusion method [42]. For this purpose, three Grampositive bacterial strains (*Staphylococcus aureus*, *Streptococcus mutans* and *Enterococcus faecalis*) were selected as well as two Gram-negative bacterial strains (*Klebsiella pneumoniae* and *Escherichia coli*). The tested derivatives inhibition zones are listed in the supplementary file, while their MIC values are listed in Table 1.

When compared with chloramphenicol as a reference drug (MIC values of 12.5 μ g/mL against all the tested strains), the starting precursors 2-carbohydrazide **9a**, bearing 6-(4-chlorophenyl)thienopyridine moiety, exhibited more effective antibacterial activities than nicotinonitrile **7a**. Hence, **9a** showed MIC values in the range of 12.5–25 μ g/mL, while **7a** showed MIC values in the range of 25–50 μ g/mL against all the tested strains. Likewise,



Scheme 3 Synthesis of 2-carbohydrazide 9a and 2-carboxylate 10



Scheme 4 Synthesis of N'-substituted benzaldehyde hydrazones 13 and 14



Scheme 5 Direct synthesis of hydrazones 13

2-carbohydrazide **9b**, bearing 6-(4-nitrophenyl)thienopyridine moiety, showed more potent antibacterial activities (MIC values of 25–50 μ g/mL) than nicotinonitrile **7b** (MIC values of 50–100 μ g/mL).

The new series of hydrazones **13**, bearing 6-(4-chlorophenyl)thienopyridine moiety, showed a diverse antibacterial activity. Hence, hydrazones **13b**, **13c** and **13d**, with *p*-Cl, *p*-Br and *p*-Me, respectively, exhibited the best antibacterial activities. These derivatives exhibited more potent or equal antibacterial activities when compared with chloramphenicol. Their MIC values were in the range of $6.2-12.5 \,\mu\text{g/mL}$.

The hydrazones **13a**, **13g** and **13k**, with p–H, p-PhCOO and p-OCH₂O, respectively, showed decreased antibacterial activities. Hence, **13g** exhibited MIC values of the range 25–50 µg/mL, while hydrazones **13a** and **13k** exhibited MIC values of the range 50–100 µg/mL.

Moreover, hydrazones **13i** and **13j**, with p-NO₂ and p-EtOCO, as well as **13e**, **13f** and **13h**, with p-OH, p-MeO and p-NMe₂, respectively, exhibited mild or poor antibacterial activities with MIC values of 100–200 µg/mL, when compared with the reference drug.

On the other hand, hydrazones 14, bearing 6-(4-nitrophenyl)thienopyridine moiety, showed less effective antibacterial activities than hydrazones 13, bearing 6-(4-chlorophenyl)thienopyridine moiety. Hence, hydrazones 14b, 14c and 14d, with *p*-Cl, *p*-Br and *p*-Me, respectively, exhibited MIC values of the range 25–50 µg/mL, while hydrazones 14a, with *p*-H, exhibited MIC values of 100–200 µg/mL, when compared with the reference drug. In addition, 14e, and 14f, with *p*-OH, and *p*-MeO, respectively, exhibited poor antibacterial activities.

The substitution pattern of **13**, bearing 6-(4-chlorophenyl) moiety, was carefully chosen to provide diverse electronic environment to the target derivatives. Therefore, electron releasing substituents such as *p*-Me, *p*-MeO, *p*-OH and *p*-NMe₂ groups and electron-withdrawing substituents, such as *p*-Cl, *p*-Br, *p*-NO₂, *p*-PhCOO and *p*-EtOCO groups, were selected as substituents on the skeleton of hydrazones **13a–13k**. Experimental results revealed differences in the in vitro antibacterial activities between *p*-substituted benzaldehyde hydrazones **13** indicating the effect of substituents on the resulting activity.

Table 1MIC values ofnew hydrazones, bearing6-(4-chlorophenyl)thienopyridine moiety

Compound MIC (µg/mL) Gram-positive bacteria Gram-negative bacteria S. aureus S. mutans E. faecalis K. pneumoniae E. coli 25 7a 50 25 25 50 7b 50 100 50 50 100 9a 12.5 25 12.5 12.5 25 9b 25 50 50 25 50 13a 50 50 100 100 100 13b 6.2 12.5 6.2 12.5 6.2 12.5 12.5 6.2 6.2 12.5 13c 12.5 13d 6.2 6.2 12.5 12.5 13e -ve -ve 200 200 -ve 13f 200 -ve 200 -ve -ve 25 25 25 50 50 13g 13h -ve 200 -ve -ve 200 200 200 13i 100 100 200 13j 100 100 100 100 200 13k 50 50 100 50 100 14a 100 100 200 200 200 14b 25 50 25 50 25 14c 50 50 25 25 25 14d 25 25 50 25 50 14e -ve -ve -ve -ve -ve 14f -ve -ve -ve -ve -ve

In order to elucidate the relationship between electronic properties of the hydrazones **13** and their antibacterial activities, we compared the values of electronic substituent constant (σ_p), field constant (F), resonance effect constant (R) [43] with the MIC values against both *Staphylococcus aureus* (bacteria) and *Klebsiella pneumoniae* as examples of Gram-positive and Gram-negative bacterial strains, respectively (Table 2). After examining Table 2, no relation was

Chloramphenicol

12.5

12.5

observed between values of MIC and σ_p or *F*. On the other hand, a clear relation was observed between the values of *R* and MIC. It is obvious that resonance effect constant (*R*) values of electron releasing Cl (*R*=-0.19), Br (*R*=-0.22) and Me (*R*=-0.18) groups are very close. The hydrazones **13b**, **13c** and **13d** with these substituents are the most potent antibacterial agents with lower or equal MIC values when compared with the reference drug (Table 2).

12.5

12.5

12.5

Compound	Substituent	$\sigma_{ m p}$	F	R	MIC (µg/mL)	
					S. aureus	K. pneumoniae
1 3 a	Н	0.00	0.03	0.00	50	100
13b	Cl	0.23	0.44	-0.19	6.2	12.5
13c	Br	0.23	0.45	-0.22	12.5	6.2
13d	Me	-0.17	0.01	-0.18	6.2	12.5
13e	OH	-0.37	0.33	-0.70	-ve	200
13f	MeO	-0.27	0.29	-0.56	200	-ve
13g	PhCOO	0.13	0.26	-0.13	25	25
13h	NMe ₂	-0.83	0.15	-0.98	-ve	-ve
13i	NO ₂	0.78	0.65	0.13	100	100
13j	EtOCO	0.45	0.34	0.11	100	100
13k	OCH ₂ O	-0.16	-0.11	-0.05	50	50

Table 2The relation betweenelectronic constants of newhydrazones13 and their MICvalues

Table 2 reveals that as the resonance effect constant (R) for the substituent attached to hydrazone become more +ve, the antibacterial activity decreased. This may be attributed to the electron density of the hydrazone. Since, electron releasing group has increased the electron density making the target hydrazone effective against different bacterial strains [44].

However, Table 2 also reveals that hydrazones attached to strong electron substituents exhibited decreased antibacterial potency. Therefore, hydrazones **13e**, **13f** and **13h** that attached to OH (R = -0.70), MeO (R = -0.56) and NMe₂ (R = -0.98) groups exhibited poor or fair antibacterial activities. This may be attributed to too high electron density resulted in more difficult diffusion through the bacteria cells, and so, substantial activity loss may occur [45].

On the other hand, experimental findings showed the reliance of the antibacterial activities on the substitution at para-position of 6-aryl group on the skeleton of thienopyridine. Therefore, hydrazones **14a–14f**, linked to more electron-withdrawing 6-(4-nitrophenyl) group, demonstrated less effective antibacterial activity than their analogues **13a–13f**, linked to more electron-donating 6-(4-chlorophenyl) group.

Likewise, we observed the dependence of antibacterial activities of hydrazones 14 on the values of resonance effect constant (R). The relation between R and the MIC values of hydrazones 14a–14f against both *Staphylococcus aureus* and *Klebsiella pneumoniae* is described in Table 3.

Among the new series of hydrazones 14, compounds 14b, 14c and 14d (R values from -0.18 to -0.22) had the best antibacterial potencies. Such derivatives showed MIC values of 25–50 µg/mL (Table 3) [41]. In addition, hydrazones 14e and 14f linked to OH (R = -0.70) and MeO (R = -0.56) moieties exhibited fair antibacterial activities [45].

As a summary, synthesis of effective antibacterial hydrazones, bearing thienopyridine moiety, could be accomplished by optimizing the electron density of the substituents on their structure [46].

Cytotoxicity against eukaryotic cells

Several hydrazones, carrying thienopyridine moiety, **13a**, **13b**, **13c**, **13d**, **13g**, **13k**, **14b**, **14c** and **14d** have been reported for in vitro cytotoxic activities against each human breast carcinoma cell line (MCF-7), colon cancer cell line (Caco2) and liver hepatocellular carcinoma cell line (HEPG2) (Table 4). The cytotoxicity findings are expressed as the concentration of the tested hydrazone **13** needed to inhibit 50 percent of cell growth (IC₅₀, μ g/mL).

The hydrazones **14b**, **14c** and **14d** were found to be noncytotoxic to all of the cell lines tested. The cell viability percentage of different cell lines was evaluated after treatment with hydrazones **14** and the reference Doxorubicin for 24 h (see the supplementary file). The viability of all the tested cells was > 70 percent when the cells were treated with concentrations of up to 75 µg/mL of these hydrazones (lowest viability value defined by ISO 10993-5 [47] to find a substance as non-cytotoxic).

Among the hydrazones 13, compound 13d exhibited the strongest cytotoxic effect against MCF-7 with $IC_{50} = 18.03 \pm 0.5 \mu g/mL$ (Fig. 1). In addition, hydrazones 13b, 13c and 13g exhibited decreased cytotoxic activities with $IC_{50} = 23.67 \pm 0.3$, 25.51 ± 0.4 and $29.37 \pm 0.3 \mu g/mL$, respectively, while the remaining hydrazones 13 exhibited mild cytotoxic activities when compared with Doxorubicin ($IC_{50} = 16.39 \pm 0.3 \mu g/mL$) (Table 4).

With regard to the cytotoxicity against colon cancer Caco2 cell line, hydrazone **13d** exhibited strong cytotoxic activity with IC₅₀=25.99±0.6 µg/mL (Fig. 2). Moreover, hydrazones **13b** and **13c** exhibited moderate activities with IC₅₀=31.09±0.4 and 32.64±0.4 µg/mL, respectively. Rest of hydrazones **13** exhibited mild cytotoxic activities when compared with Doxorubicin (IC₅₀=25.30±0.4 µg/mL) (Table 4).

With regard to the cytotoxicity against liver hepatocellular carcinoma HEPG2 cell line, both hydrazones **13b** and **13c** exhibited strong cytotoxic activity with $IC_{50} = 19.48 \pm 0.5$

Table 3 The relation between resonance effect constant (R) of new hydrazones **14** and their MIC values

Compound	Substituent	R	MIC (µg/mL)		
			S. aureus	K. pneumoniae	
14a	Н	0.00	100	200	
14b	Cl	-0.19	25	50	
14c	Br	-0.22	50	25	
14d	Me	-0.18	25	50	
14e	OH	-0.70	-ve	-ve	
14f	MeO	-0.56	-ve	-ve	

Table 4 The cell inhibition percentage and IC_{50} values of some novel hydrazones against each of MCF-7, Caco2 and HEPG2, and using Doxorubicin as a reference drug

Compound	IC ₅₀ (µg/mL)				
	MCF-7	Caco2	HEPG2		
13a	48.02 ± 0.4	68.14 ± 0.5	64.15 ± 0.5		
13b	23.67 ± 0.3	31.09 ± 0.4	19.48 ± 0.5		
13c	25.51 ± 0.4	32.64 ± 0.4	19.30 ± 0.3		
13d	18.03 ± 0.5	25.99 ± 0.6	25.85 ± 0.4		
13g	29.37 ± 0.3	42.56 ± 0.6	31.00 ± 0.3		
13k	46.47 ± 0.4	58.51 ± 0.3	53.26 ± 0.4		
Doxorubicin	16.39 ± 0.3	25.30 ± 0.4	18.89 ± 0.3		

Fig. 1 Cell viability percentage of MCF-7 after treatment with some hydrazones **13** for 24 h



Fig. 2 Cell viability percentage of Caco2 after treatment with some hydrazones **13** for 24 h



Concentration (µg/mL)

The in vitro evaluation of cyclooxygenase-2 inhibition

The hydrazones **13a**, **13b**, **13c**, **13d**, **13g** and **13k** were tested for inhibiting cyclooxygenase-2 enzyme (COX-2) [48]. The concentration required to inhibit 50% of COX-2 enzyme (IC₅₀) is expressed in μ M. Celecoxib was used as a reference drug with IC₅₀ of 0.115 μ M (see Table 5).

Hydrazones **13c** and **13d** revealed more inhibitory activity than Celecoxib with IC_{50} of 0.110 and 0.104 μ M, respectively. Hydrazone **13b** revealed nearly inhibition activity equipotent to Celecoxib with IC_{50} of 0.121 μ M. On the other hand, hydrazones **13a**, **13g** and **13k** exhibited decreased inhibition activity against COX-2 enzyme with IC_{50} in the range of 0.133–0.182 μ M.

The in silico study: Molecular modeling

In comparison with Celecoxib, molecular docking was performed to test the capability of some new hydrazones **13** as potential COX-2 inhibitors. Thus, compounds **13b**,

Table 5 The IC_{50} values in μM of some hydrazones against COX-2 enzyme. Celecoxib was used as the standard drug		IC ₅₀ (μM) against COX-2
	13 a	0.182
	13b	0.121
	13c	0.110
	13d	0.104
	13g	0.133
	13k	0.165
	Celecoxib	0.115

13c and **13d** as well as Celecoxib were docked as ligand molecules with COX-2 (PDB ID: 4cox) as the target protein to achieve their optimum conformation, with reduced free energy [49, 50]. Computational docking studies have been used to estimate the binding energies of the interactions between each ligand and the amino acid residues in the target enzyme [51]. The findings are shown in Table 6.

Figure 4 displays 2D and 3D ligand interactions of Celecoxib with the target 4cox. It showed a large network of hydrogen bonding interactions with residues of GLU 46, CYS 47, TYR 136 and LYS 137 amino acids in the target enzyme with binding distances and energies varying from -0.6 to 5.6 kcal/mol and from 2.92 to 3.36 Å.



of HEPG2 after treatment with some hydrazones **13** for 24 h

Fig. 3 Cell viability percentage

Table 6Energy values obtainedin docking calculations of newhydrazones 13 with COX-2(PDB ID: 4cox)

Compound Distance (Å) Ligand moiety Site Interaction E (kcal/mol) 13b N 48 O CYS 47 (A) H-donor 2.80-1.5O 12 N CYS 47 (A) H-acceptor 2.72 -5.4 CA GLN 42 (A) Pi-H -3.23.56 6-ring CA MET 48 (A) Pi-H -1.1 5-ring 3.61 13c S 10 O CYS 41 (A) H-donor 2.84 -0.7S 23 O ALA 151 (A) H-donor -1.7 2.67 O 12 N CYS 47 (A) H-acceptor 2.59 -5.75-ring CG ARG 44 (A) Pi-H 3.34 -1.46-ring CA GLU 46 (A) Pi-H 2.98 -3.713d S 10 -0.7O ARG 44 (A) H-donor 3.15 N 13 O ARG 44 (A) H-donor 2.84 -4.2O 12 OH TYR 130 (A) H-acceptor 2.42 -6.2-0.7N 15 OH TYR 130 (A) H-acceptor 3.11 -0.8Pi-H 6-ring CA GLU 46 (A) 3.85 6-ring N CYS 47 (A) Pi-H 3.09 -3.8Celecoxib N 19 OE1 GLU 46 (A) H-donor 3.11 -5.6N 19 O CYS 47 (A) H-donor 2.92 -2.5O 18 CA TYR 136 (A) -0.6H-acceptor 3.28 O 18 NZ LYS 137 (A) H-acceptor -0.83.36



Fig. 4 The 2D and 3D ligand interactions of Celecoxib with COX-2 (4cox)

Experimental results demonstrated capability of hydrazone **13d** as a potent COX-2 inhibitor. Docking study of compound **13d** revealed more strong binding interactions with COX-2 than the reference Celecoxib (see Fig. 5). The figure revealed a strong network of hydrogen bonding interactions of both hydrazone-NH and thienopyridine-S with residue of ARG 44 (2.84 Å, -4.2 kcal/mol; 3.15 Å, -0.7 kcal/mol, respectively) as well as between both carbonyl-O and hydrazone-N with residue of TYR 130 (2.42 Å, -6.2 kcal/mol; 3.11 Å, -0.7 kcal/mol, respectively). In addition, it showed strong π -H interactions between hydrazone-aryl moieties with residues of both CYS 47 and GLU 46 (3.09 Å, -3.8 kcal/mol; 3.85 Å, -0.8 kcal/mol, respectively).

Hydrazone **13c** showed good binding interactions with COX-2 (see supplementary file). Its docking poses



Fig. 5 The 2D and 3D ligand interactions of hydrazone 13d with COX-2 (4cox)

revealed a network of hydrogen bonding interactions of carbonyl-O with residue of CYS 47 (2.59 Å, -5.7 kcal/mol), thienopyridine-S with residue of CYS 41 (2.84 Å, -0.7 kcal/mol) and thiophene–S with residue of ALA 151 (2.67 Å, -1.7 kcal/mol). Also, it showed strong π –H interactions between hydrazone-aryl moiety with residue of GLU 46 (2.98 Å, -3.7 kcal/mol) and thiophene ring with residue of ARG 44 (3.34 Å, -1.4 kcal/mol).

Furthermore, hydrazone **13b** showed decreased binding interactions with COX-2 when compared with Celecoxib (see supplementary file). Its docking poses demonstrated a network of hydrogen bonding interactions of both carbonyl-O and amino-N with residue of CYS 47 (2.72 Å, -5.4 kcal/mol; 2.80 Å, -1.5 kcal/mol, respectively). Also, it showed strong π -H interactions between hydrazone-aryl moiety with residue of GLN 42 (3.56 Å, -3.2 kcal/mol) and thiophene ring with residue of MET 48 (3.61 Å, -1.1 kcal/mol).

As a summary, both experimental and docking studies could predict the promising potential of the hydrazones being tested as effective COX-2 inhibitors. Docking poses of these hydrazones showed the relation between the electronic properties of the hydrazones and their inhibitory activities. Therefore, hydrazones **13b**, **13c**, and **13d**, linked to aryl group with *p*-Cl, *p*-Br and *p*-Me, respectively, revealed favorable hydrophobic interactions between hydrazone-aryl moieties with amino acids residues of COX-2 (energy of -3.2 to -3.8 kcal/mol). In addition, docking poses of these hydrazones demonstrated strong networks of hydrogen bonding interactions of carbonyl-O with COX-2 amino acids residues (energy of -5.4 to -6.2 kcal/mol).

Conclusion

The starting intermediate carbohydrazide was prepared and used to prepare a series the target hydrazones incorporating thienopyridine moiety. The in vitro antibacterial potency of the target hydrazones was evaluated against different strains of Gram-positive and Gram-negative bacteria. The hydrazones **13b**, **13c** and **13d**, with *p*-Cl, *p*-Br and *p*-Me, respectively, exhibited the best antibacterial potencies against all the tested bacteria. The in vitro cytotoxicity of some novel hydrazones was tested against several eukaryotic cell lines. The hydrazones **13b**, **13c** and **13d** exhibited the best cytotoxicity results against all the tested cells. Furthermore, both experimental and docking studies could predict the promising inhibitory activities of hydrazones **13c** and **13d** against COX-2 enzyme, when compared with Celecoxib.

Experimental

Introduction

All solvents were acquired from commercial sources and used as received unless otherwise stated. All other chemicals were acquired from Merck or Aldrich. These chemicals were used without further purification. The melting points were measured on a Stuart melting point apparatus and are uncorrected. IR spectra were recorded on a Smart iTR, which is an ultra-high-performance, versatile attenuated total reflectance (ATR) sampling accessory on the Nicolet iS10 FT-IR spectrometer manufactured. NMR spectra were recorded on Bruker Avance III 400 MHz spectrophotometer (400 MHz for ¹H and 100 MHz for ¹³C) using TMS as an internal standard and DMSO- d_6 as solvent, and chemical shifts were expressed as δ ppm units. Mass spectra were recorded on a GC–MS-QP1000EX spectrometer using inlet type at 70 eV. Elemental analyses were carried out on a EuroVector instrument C, H, N analyzer EA3000 Series.

Methods and spectral data

Synthesis of pyridine-2(1H)-thiones 5

A binary mixture of carbonyl derivative 3 (5 mmol) and 2-cyanothioacetamide 4 (5 mmol) in ethanol (15 mL) in the presence of piperidine (0.3 mL) was boiled at reflux for 4 h. The reaction mixture was cooled, filtered off, washed with ethanol, and then, the product was recrystallized from the proper solvent.

6-(4-Chlorophenyl)-4-(thiophen-2-yl)-2-thioxo-1,2-dihydropyridine-3-carbonitrile (5a) Pale orange solid (ethanol, 71%); m.p. 286–287 °C; IR ($v \text{ cm}^{-1}$): 3155 (NH), 2218 (CN);¹H-NMR (DMSO- d_6): δ 7.38 (t, 1H, thiophene-H4), 7.43 (d, 2H, Ar–H's), 8.04 (d, 1H, thiophene-H5), 8.08–8.11 (m, 3H, pyridine-H5, 2 Ar–H's), 8.17 (d, 1H, thiophene-H3), 14.08 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): 109.6, 111.7 (pyridine-C3, C5), 117.6 (CN), 128.7, 128.9, 129.0, 130.4, 133.1, 135.1, 135.7, 136.4 (Ar–C's and thiophene-C's), 147.8, 152.4, 180.2 (pyridine-C4, C6, C2); MS m/z (%): 329 (M⁺, 28.5), 331 (M⁺+2, 9.9); Anal. for C₁₆H₉ClN₂S₂: C, 58.44; H, 2.76; N, 8.52; found: C, 58.30; H, 2.89; N, 8.64%.

6-(4-Nitrophenyl)-4-(thiophen-2-yl)-2-thioxo-1,2-dihydropyridine-3-carbonitrile (5b) Pale orange solid (dioxane/ ethanol mixture, 74%); m.p. above 300 °C; IR (ν cm⁻¹): 3158 (NH), 2219 (CN);¹H-NMR (DMSO- d_6): δ 7.32 (t, 1H, thiophene-H4), 7.98 (d, 1H, thiophene-H5), 8.04 (d, 2H, Ar–H's), 8.10 (s, 1H, pyridine-H5), 8.15 (d, 1H, thiophene-H3), 8.41 (d, 2H, Ar–H's), 14.22 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): 109.2, 111.6 (pyridine-C3, C5), 117.2 (*C*N), 121.9, 124.2, 128.4, 128.7, 132.8, 136.2, 138.6, 144.5 (Ar– *C*'s and thiophene-*C*'s), 146.8, 152.7, 180.6 (pyridine-*C*4, *C*6, *C*2); Anal. for C₁₆H₉N₃O₂S₂ (339.3): C, 56.62; H, 2.67; N, 12.38; found: C, 56.51; H, 2.54; N, 12.48%.

Synthesis of nicotinonitriles 7

A mixture of 2-thioxopyridine 5 (5 mmol) and ethyl chloroacetate 6 (5 mmol) in ethanol (15 mL) in the presence of sodium acetate (5 mmol) was stirred at rt for 1 h. The product that formed was collected by filtration, washed with water, dried and then recrystallized from the proper solvent.

Ethyl 2-((6-(4-chlorophenyl)-3-cyano-4-(thiophen-2-yl) pyridin-2-yl)thio)acetate (7a) Colorless solid (ethanol/dioxane mixture, 95%); m.p. 200–202 °C;; IR (ν cm⁻¹): 2214 (CN), 1729 (CO); ¹H-NMR (DMSO- d_6): δ 1.14 (t, 3H, CH₂CH₃), 4.10 (q, 2H, CH₂CH₃), 4.24 (s, 2H, CH₂), 7.34 (t, 1H, thiophene-H4), 7.60 (d, 2H, Ar–H's), 7.97–8.02 (m, 3H, pyridine-H5, thiophene-H5 and H3), 8.24 (d, 2H, Ar– H's); ¹³C-NMR (DMSO- d_6): δ 14.4 (CH₃), 33.1 (SCH₂), 61.6 (OCH₂), 100.2, 114.3 (pyridine-C3, C5), 116.2 (CN), 127.8, 129.1, 129.9, 130.2, 130.9, 135.0, 135.8, 136.7 (Ar– C's and thiophene-C's), 146.1, 158.2, 162.7 (pyridine-C4, C6, C2), 169.0 (CO); MS *m*/*z* (%): 414 (M⁺, 40.9), 416 (M⁺+2, 14.0); Anal. for C₂₀H₁₅CIN₂O₂S₂: C, 57.90; H, 3.64; N, 6.75; found: C, 58.02; H, 3.53; N, 6.81%.

Ethyl 2-((3-cyano-6-(4-nitrophenyl)-4-(thiophen-2-yl)pyridin-2-yl)thio)acetate (7b) Colorless solid (dioxane, 90%); m.p. 224–225 °C;; IR ($v \text{ cm}^{-1}$): 2214 (CN), 1728 (CO); ¹H-NMR (DMSO- d_6): δ 1.13 (t, 3H, CH₂CH₃), 4.10 (q, 2H, CH₂CH₃), 4.21 (s, 2H, CH₂), 7.37 (t, 1H, thiophene-H4), 7.95 (s, 1H, pyridine-H5), 7.99 (d, 1H, thiophene-H5), 8.06 (d, 1H, thiophene-H3), 8.21 (d, 2H, Ar–H's), 8.33 (d, 2H, Ar–H's); ¹³C-NMR (DMSO- d_6): δ 14.3 (CH₃), 33.2 (SCH₂), 61.6 (OCH₂), 100.1, 114.2 (pyridine-C3, C5), 116.3 (CN), 122.2, 124.5, 127.7, 129.9, 130.8, 136.5, 138.8, 144.8 (Ar– C's and thiophene-C's), 146.0, 157.7, 162.5 (pyridine-C4, C6, C2), 169.1 (CO); Anal. for C₂₀H₁₅N₃O₄S₂ (425.4): C, 56.46; H, 3.55; N, 9.88; found: C, 56.31; H, 3.42; N, 10.06%.

Synthesis of thieno[2,3-b]pyridine-2-carbohydrazides 9

A mixture of nicotinonitrile **7** or thienopyridine **10** (5 mmol) and hydrazine hydrate (5 mL, 80%) in pyridine (15 mL) was boiled at reflux for 5–8 h. The product that formed was collected by filtration, washed with ethanol, dried and then recrystallized from the proper solvent.

3-Amino-6-(4-chlorophenyl)-4-(thiophen-2-yl)thieno[2,3-b] pyridine-2-carbohydrazide (9a) Yellow solid (ethanol/ dioxane mixture, 77%); m.p. 250–251 °C; IR ($v \text{ cm}^{-1}$): 3396, 3344, 3188 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 4.51 (br s, 2H, NHN H_2), 6.11 (br s, 2H, NH₂), 7.28 (t, 1H, thiophene-H4), 7.42 (d, 1H, thiophene-H5), 7.53 (d, 2H, Ar–H's), 7.72 (s, 1H, pyridine-H5), 7.84 (d, 1H, thiophene-H3), 8.22 (d, 2H, Ar–H's), 9.15 (br s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 94.2, 118.9, 121.8 (thienopyridine-*C*2, *C*5, *C*3a), 127.9, 128.5, 128.9, 129.4, 130.6, 134.7, 135.9, 136.4 (Ar–*C*'s and thiophene-*C*'s), 139.9, 145.1 (2*C*), 153.8 (thienopyridine-*C*3, *C*4, *C*6, *C*7a), 164.4 (thienopyridine-*CO*); MS m/z (%): 400 (M⁺, 20.4), 402 (M⁺+2, 8.3); Anal. for C₁₈H₁₃ClN₄OS₂: C, 53.93; H, 3.27; N, 13.98; found: C, 53.84; H, 3.41; N, 14.09%.

3-Amino-6-(4-nitrophenyl)-4-(thiophen-2-yl)thieno[2,3-*b***] pyridine-2-carbohydrazide (9b) Yellow solid (dioxane, 81%); m.p. 259–262 °C; IR (v \text{ cm}^{-1}): 3468, 3313, 3255 (NH, NH₂); ¹H-NMR (DMSO-***d***₆): \delta 4.42 (br s, 2H, NHN***H***₂), 6.14 (br s, 2H, NH₂), 7.30 (t, 1H, thiophene-H4), 7.40 (d, 1H, thiophene-H5), 7.69 (s, 1H, pyridine-H5), 7.82 (d, 1H, thiophene-H3), 8.14 (d, 2H, Ar–H's), 8.31 (d, 2H, Ar–H's), 9.20 (br s, 1H, NH); ¹³C-NMR (DMSO-***d***₆): \delta 94.5, 118.5, 121.5 (thienopyridine-***C***2,** *C***5,** *C***3a), 122.1, 124.0, 127.7, 128.6, 130.7, 136.3, 138.5 (Ar–***C***'s and thiophene-***C***'s), 139.5 (thienopyridine-***C***3), 144.4 (Ar–***C***), 144.9, 145.2, 153.4 (thienopyridine-***C***4,** *C***6,** *C***7a), 164.2 (thienopyridine-***CO***); Anal. for C₁₈H₁₃N₅O₃S₂ (411.4): C, 52.54; H, 3.18; N, 17.02; found: C, 52.62; H, 3.22; N, 16.87%.**

Synthesis of ethyl 3-amino-6-(4-chlorophenyl)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carboxylate (**10**)

A mixture of nicotinonitrile **7** (5 mmol) and ethyl chloroacetate **6** (5 mmol) in ethanol (20 mL) in the presence of sodium metal (5 mmol) was heated at reflux for 2 h. The products that formed were collected by filtration, washed with ethanol, dried and then recrystallized from dioxane as yellow solid (93%); m.p. 160–161 °C; IR (v cm-1): 3462, 3333 (NH₂), 1664 (CO); ¹H-NMR (DMSO- d_6): δ 1.30 (t, 3H, CH₂CH₃), 4.28 (q, 2H, CH₂CH₃), 6.11 (s, 2H, NH₂), 7.31 (t, 1H, thiophene H-4), 7.46 (d, 1H, thiophene-H5), 7.56 (d, 2H, Ar–H's), 7.89 (s, 1H, pyridine-H5), 7.93 (d, 1H, thiophene-H3), 8.23 (d, 2H, Ar–H's); MS m/z (%): 414 (M⁺, 76.4), 416 (M⁺+2, 28.7); Anal. for C₂₀H₁₅ClN₂O₂S₂: C, 57.90; H, 3.64; N, 6.75; found: C, 57.74; H, 3.77; N, 6.92%.

General procedure for synthesis of hydrazones 13 and 14

A mixture of each of 2-carbohydrazides **9a,b** (5 mmol) and the appropriate arylidenemalononitrile **11a–11k** or the appropriate aromatic aldehydes **15a–15c** (5 mmol) in pyridine (15 mL) was boiled at reflux. The reaction was followed by TLC analyses to determine the reaction time. Then, the solvent was evaporated to its half volume and cooled. The solid product was collected by filtration, washed with ethanol, dried and then recrystallized from the proper solvent.

(*E*)-3-amino-*N*'-benzylidene-6-(4-chlorophenyl)-4-(thiophe n-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (13a) Yellow solid (ethanol/dioxane mixture); m.p. 240–242 °C; IR ($v \text{ cm}^{-1}$): 3329, 3244 (NH, NH₂); ¹H-NMR (DMSO-*d*₆): δ 6.63 (br s, 2H, NH₂), 7.32 (t, 1H, thiophene-H4), 7.46–7.50 (m, 4H, 3 Ar–H's and thiophene-H5), 7.55 (d, 2H, Ar–H's), 7.84–7.86 (m, 3H, 2 Ar–H's and pyridine-H5), 7.90 (d, 1H,

thiophene-H3), 8.12 (s, 1H, methine-H), 8.28 (d, 2H, Ar–H's), 11.49 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 94.6, 119.5, 122.2 (thienopyridine-C2, C5, C3a), 127.5, 127.9, 128.5, 128.9, 129.2, 129.4, 130.5, 130.7, 134.3, 134.7, 135.9, 136.5 (Ar–C's and thiophene-C's), 139.9 (thienopyridine-C3), 144.8 (methine-C), 145.5, 145.8, 154.4 (thienopyridine-C4, C6, C7a), 168.6 (thienopyridine-CO); Anal. for C₂₅H₁₇ClN₄OS₂ (489.0): C, 61.40; H, 3.50; N, 11.46; found: C, 61.23; H, 3.36; N, 11.62%.

(E)-3-Amino-N'-(4-chlorobenzylidene)-6-(4-chlorophenyl) -4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (13b) Yellow solid (ethanol/dioxane mixture); m.p. above 300 °C; IR (v cm⁻¹): 3322, 3240 (NH, NH₂); ¹H-NMR $(DMSO-d_6): \delta 6.64$ (br s, 2H, NH₂), 7.29 (t, 1H, thiophene-H4), 7.43 (d, 1H, thiophene-H5), 7.54 (d, 2H, Ar-H's), 7.60 (d, 2H, Ar-H's), 7.84-7.86 (m, 3H, 2 Ar-H's and pyridine-H5), 7.94 (d, 1H, thiophene-H3), 8.11 (s, 1H, methine-H), 8.27 (d, 2H, Ar-H's), 11.55 (s, 1H, NH); ¹³C-NMR (DMSO-*d*₆): δ 94.3, 119.0, 121.9 (thienopyridine-*C*2, C5, C3a), 127.8, 128.4, 128.6, 128.8, 128.9, 129.4, 130.6, 132.8, 134.5, 134.8, 135.9, 136.5 (Ar-C's and thiophene-C's), 140.2 (thienopyridine-C3), 142.9 (methine-C), 145.3, 146.2, 154.5 (thienopyridine-C4, C6, C7a), 167.9 (thienopyridine-CO); Anal. for C₂₅H₁₆Cl₂N₄OS₂ (523.4): C, 57.36; H, 3.08; N, 10.70; found: C, 57.51; H, 3.00; N, 10.58%.

(*E*)-3-Amino-*N*'-(4-bromobenzylidene)-6-(4-chlorophenyl) -4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (13c) Yellow solid (ethanol/dioxane mixture); m.p. above $300 \,^{\circ}$ C; IR ($v \, \text{cm}^{-1}$): 3322, 3240 (NH, NH₂); ¹H-NMR (DMSO-*d*₆): $\delta \, 6.66$ (br s, 2H, NH₂), 7.31 (t, 1H, thiophene-H4), 7.44 (d, 1H, thiophene-H5), 7.57–7.60 (m, 4H, Ar– H's), 7.85–7.87 (m, 3H, 2 Ar–H's and pyridine-H5), 7.93 (d, 1H, thiophene-H3), 8.10 (s, 1H, methine-H), 8.27 (d, 2H, Ar–H's), 11.52 (s, 1H, NH); Anal. for C₂₅H₁₆BrClN₄OS₂ (567.9): C, 52.87; H, 2.84; N, 9.87; found: C, 52.99; H, 2.97; N, 9.66%.

(*E*)-3-Amino-6-(4-chlorophenyl)-*N*'-(4-methylbenzylidene) -4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (13d) Yellow solid (dioxane); m.p. 280–281 °C; IR (v cm⁻¹): 3319, 3250 (NH, NH₂); ¹H-NMR (DMSO-*d*₆): δ 2.35 (s, 3H, CH₃), 6.73 (br s, 2H, NH₂), 7.29–7.32 (m, 3H, 2 Ar–H's and thiophene-H4), 7.47 (d, 1H, thiophene-H5), 7.55 (d, 2H, Ar–H's), 7.63 (d, 2H, Ar–H's), 7.82 (s, 1H, pyridine-H5), 7.93 (d, 1H, thiophene-H3), 8.10 (s, 1H, methine-H), 8.25 (d, 2H, Ar–H's), 11.41 (s, 1H, NH); Anal. for C₂₆H₁₉ClN₄OS₂ (503.0): C, 62.08; H, 3.81; N, 11.14; found: C, 61.95; H, 3.74; N, 11.26%.

(*E*)-3-Amino-6-(4-chlorophenyl)-*N*'-(4-hydroxybenzylidene)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide

(13e) Yellow solid (dioxane); m.p. above 300 °C; IR ($v \text{ cm}^{-1}$): 3445 (OH), 3318, 3252 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 6.72 (br s, 2H, NH₂), 6.91 (d, 2H, Ar–H's), 7.33 (t, 1H, thiophene-H4), 7.45 (d, 1H, thiophene-H5), 7.57 (d, 2H, Ar–H's), 7.66 (d, 2H, Ar–H's), 7.83 (s, 1H, pyridine-H5), 7.93 (d, 1H, thiophene-H3), 8.06 (s, 1H, methine-H), 8.27 (d, 2H, Ar–H's), 10.02 (br s, 1H, OH), 11.44 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 95.0 (thienopyridine-*C*2), 115.7 (Ar–*C*), 118.2, 121.5 (thienopyridine-*C*5, *C*3a), 124.9, 127.8, 128.5, 128.7, 129.1, 129.4, 130.5, 134.8, 135.8, 136.6 (Ar–*C*'s and thiophene-*C*'s), 140.3 (thienopyridine-*C*3), 144.6 (methine-*C*), 145.1, 145.9, 155.2 (thienopyridine-*C*4, *C*6, *C*7a), 159.4 (Ar–*C*), 167.8 (thienopyridine-*C*O); Anal. for C₂₅H₁₇ClN₄O₂S₂ (505.0): C, 59.46; H, 3.39; N, 11.09; found: C, 59.31; H, 3.53; N, 10.87%.

(E)-3-Amino-6-(4-chlorophenyl)-N'-(4-methoxybenzylidene)-4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (13f) Yellow solid (ethanol/dioxane mixture); m.p. 284 °C; IR $(v \text{ cm}^{-1})$: 3310, 3251 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 3.83 (s, 3H, OCH₃), 6.71 (br s, 2H, NH₂), 7.11 (d, 2H, Ar-H's), 7.31 (t, 1H, thiophene-H4), 7.46 (d, 1H, thiophene-H5), 7.57 (d, 2H, Ar-H's), 7.77 (d, 2H, Ar-H's), 7.86 (s, 1H, pyridine-H5), 7.91 (d, 1H, thiophene-H3), 8.04 (s, 1H, methine-H), 8.27 (d, 2H, Ar–H's), 11.43 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 55.2 (OCH₃), 94.8 (thienopyridine-C2), 114.2 (Ar-C), 118.3, 121.8 (thienopyridine-C5, C3a), 126.5, 127.8, 128.5, 128.6, 129.1, 129.5, 130.6, 134.8, 135.7, 136.4 (Ar-C's and thiophene-C's), 140.5 (thienopyridine-C3), 144.2 (methine-C), 145.3, 146.2, 155.5 (thienopyridine-C4, C6, C7a), 160.8 (Ar-C), 167.9 (thienopyridine-CO); Anal. for C₂₆H₁₉ClN₄O₂S₂ (519.0): C, 60.17; H, 3.69; N, 10.79; found: C, 60.29; H, 3.75; N, 10.95%.

(*E*)-4-((2-(3-Amino-6-(4-chlorophenyl)-4-(thiophen-2 -yl)thieno[2,3-*b*]pyridine-2-carbonyl)hydrazineylidene) methyl)phenyl benzoate (13g) Brown solid (dioxane); m.p. above 300 °C; IR ($v \text{ cm}^{-1}$): 3327, 3243 (NH, NH₂), 1739 (CO); ¹H-NMR (DMSO-*d*₆): δ 6.68 (br s, 2H, NH₂), 7.30–7.33 (m, 3H, 2 Ar–H's and thiophene-H4), 7.44–7.48 (m, 3H, 2 Ar–H's and thiophene-H5), 7.55–7.58 (m, 4H, Ar–H's), 7.65 (t, 1H, Ar–H's), 7.87 (s, 1H, pyridine-H5), 7.92 (d, 1H, thiophene-H3), 8.01 (s, 1H, methine-H), 8.11 (d, 2H, Ar–H's), 8.28 (d, 2H, Ar–H's), 11.48 (s, 1H, NH); Anal. for C₃₂H₂₁ClN₄O₃S₂ (609.1): C, 63.10; H, 3.48; N, 9.20; found: C, 63.26; H, 3.53; N, 9.06%.

(*E*)-3-Amino-6-(4-chlorophenyl)-*N*'-(4-(dimethylamino) benzylidene)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (13h) Brown solid (dioxane); m.p. 297–299 °C; IR ($v \text{ cm}^{-1}$): 3326, 3241 (NH, NH₂); ¹H-NMR (DMSO-*d*₆): δ 2.97 (s, 6H, N(CH₃)₂), 6.65 (br s, 2H, NH₂), 6.84 (d, 2H, Ar–H's), 7.31 (t, 1H, thiophene-H4), 7.44 (d, 1H, thiophene-H5), 7.63–7.66 (m, 4H, Ar–H's), 7.85 (s, 1H, pyridine-H5), 7.91 (d, 1H, thiophene-H3), 8.07 (s, 1H, methine-H), 8.25 (d, 2H, Ar–H's), 11.49 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 39.8 (N(CH₃)₂), 94.7 (thienopyridine-C2), 111.7 (Ar–C), 118.2 (thienopyridine-C5), 121.1 (Ar–C), 121.6 (thienopyridine-C3a), 127.7, 128.2, 128.6, 129.1, 129.5, 130.4, 134.6, 135.8, 136.5 (Ar–C's and thiophene-C's), 140.3 (thienopyridine-C3), 145.2 (methine-C), 145.4, 145.9 (thienopyridine-C4, C6), 149.3 (Ar–C), 155.4 (thienopyridine-C7a), 167.5 (thienopyridine-CO); Anal. for C₂₇H₂₂ClN₅OS₂ (532.0): C, 60.95; H, 4.17; N, 13.16; found: C, 60.95; H, 4.17; N, 13.16%.

(*E*)-3-Amino-6-(4-chlorophenyl)-*N*'-(4-nitrobenzylidene)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (13i) Orange solid (dioxane); m.p. 184–187 °C; IR (v cm⁻¹): 3329, 3237 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 6.68 (br s, 2H, NH₂), 7.31 (t, 1H, thiophene-H4), 7.47 (d, 1H, thiophene-H5), 7.59 (d, 2H, Ar–H's), 7.82 (s, 1H, pyridine-H5), 7.93 (d, 1H, thiophene-H3), 8.05 (d, 2H, Ar–H's), 8.12 (s, 1H, methine-H), 8.26 (d, 2H, Ar–H's), 8.38 (d, 2H, Ar–H's), 11.52 (s, 1H, NH); Anal. for C₂₅H₁₆ClN₅O₃S₂ (534.0): C, 56.23; H, 3.02; N, 13.12; found: C, 56.08; H, 3.14; N, 13.00%.

Ethyl (*E*)-4-((2-(3-amino-6-(4-chlorophenyl)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbonyl)hydrazineylidene) methyl)benzoate (13j) Orange solid (dioxane); m.p. 242–243 °C; IR ($v \text{ cm}^{-1}$): 3329, 3237 (NH, NH₂); ¹H-NMR (DMSO-*d₆*): δ 1.30 (t, 3H, CH₂CH₃), 4.28 (q, 2H, CH₂CH₃), 6.74 (br s, 2H, NH₂), 7.33 (t, 1H, thiophene-H4), 7.47 (d, 1H, thiophene-H5), 7.57–7.60 (m, 4H, Ar–H's), 7.82 (s, 1H, pyridine-H5), 7.90 (d, 1H, thiophene-H3), 8.00 (d, 2H, Ar–H's), 8.09 (s, 1H, methine-H), 8.24 (d, 2H, Ar–H's), 11.44 (s, 1H, NH); Anal. for C₂₈H₂₁ClN₄O₃S₂ (561.0): C, 59.94; H, 3.77; N, 9.99; found: C, 60.13; H, 3.81; N, 9.85%.

(E)-3-Amino-N'-(benzo[d] [1, 3] dioxol-5-ylmethylene)-6-(4-c hlorophenyl)-4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (13k) Yellow solid (ethanol/dioxane mixture); m.p. above 300 °C; IR ($v \text{ cm}^{-1}$): 3322, 3253 (NH, NH₂); ¹H-NMR (DMSO-*d*₆): δ 6.12 (s, 3H, OCH₂O), 6.62 (br s, 2H, NH₂), 7.06 (d, 1H, piperonal-H7), 7.22 (d, 1H, piperonal-H6), 7.31 (t, 1H, thiophene-H4), 7.44-7.46 (m, 2H, piperonal-H4 and thiophene-H5), 7.55 (d, 2H, Ar-H's), 7.87 (s, 1H, pyridine-H5), 7.92 (d, 1H, thiophene-H3), 8.01 (s, 1H, methine-H), 8.25 (d, 2H, Ar-H's), 11.45 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 94.5 (thienopyridine-C2), 101.2 (OCH₂O), 105.2, 108.3 (Ar-C), 118.1, 122.4 (thienopyridine-C5, C3a), 123.1, 127.6, 128.3, 128.6, 128.9, 129.4, 130.6, 134.7, 135.7, 136.5 (Ar-C's and thiophene-C's), 140.3 (thienopyridine-C3), 143.9 (methine-C), 145.2, 146.1 (thienopyridine-C4, C6), 147.9, 149.3 (Ar-C), 154.8 (thienopyridine-*C*7a), 168.0 (thienopyridine-*C*O); Anal. for $C_{26}H_{17}ClN_4O_3S_2$ (533.0): C, 58.59; H, 3.21; N, 10.51; found: C, 58.67; H, 3.12; N, 10.72%.

(E)-3-Amino-N'-benzylidene-6-(4-nitrophenyl)-4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (14a) Orange solid (dioxane); m.p. 266–267 °C; IR (v cm⁻¹): 3464, 3271 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 6.68 (br s, 2H, NH₂), 7.27 (t, 1H, thiophene-H4), 7.40 (d, 1H, thiophene-H5), 7.47-7.54 (m, 3H, Ar-H's), 7.84 (s, 1H, pyridine-H5), 7.88 (d, 2H, Ar-H's), 7.96 (d, 1H, thiophene-H3), 8.10 (s, 1H, methine-H), 8.17 (d, 2H, Ar-H's), 8.35 (d, 2H, Ar-H's), 11.44 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 95.2, 119.2 (thienopyridine-C2, C5), 122.3 (Ar-C), 122.7 (thienopyridine-C3a), 124.1, 127.4, 127.7, 128.3, 129.2, 130.3, 130.6, 134.5, 136.4, 138.3 (Ar-C's and thiophene-C's), 139.6 (thienopyridine-C3), 144.2 (Ar-C), 144.9 (methine-C), 145.4, 145.7, 153.8 (thienopyridine-C4, C6, C7a), 168.2 (thienopyridine-CO); Anal. for $C_{25}H_{17}N_5O_3S_2$ (499.5): C, 60.11; H, 3.43; N, 14.02; found: C, 60.03; H, 3.30; N, 14.28%.

(E)-3-Amino-N'-(4-chlorobenzylidene)-6-(4-nitrophenyl)-4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (14b) Yellow solid (dioxane); m.p. above 300 °C; IR (v cm⁻¹): 3336, 3242 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 6.58 (br s, 2H, NH₂), 7.29 (t, 1H, thiophene-H4), 7.42 (d, 1H, thiophene-H5), 7.58 (d, 2H, Ar-H's), 7.81 (s, 1H, pyridine-H5), 7.87 (d, 2H, Ar-H's), 7.95 (d, 1H, thiophene-H3), 8.09 (s, 1H, methine-H), 8.16 (d, 2H, Ar-H's), 8.36 (d, 2H, Ar-H's), 11.47 (s, 1H, NH); ¹³C-NMR (DMSO-*d*₆): δ 94.7, 119.4 (thienopyridine-C2, C5), 122.3 (Ar-C), 122.8 (thienopyridine-C3a), 124.2, 127.6, 128.5, 128.7, 128.9, 130.4, 132.6, 134.2, 136.3, 138.4 (Ar-C's and thiophene-C's), 141.3 (thienopyridine-C3), 143.6 (methine-C), 144.2 (Ar-C), 145.4, 145.9, 154.1 (thienopyridine-C4, C6, C7a), 168.1 (thienopyridine-CO); Anal. for $C_{25}H_{16}CIN_5O_3S_2$ (534.0): C, 56.23; H, 3.02; N, 13.12; found: C, 56.08; H, 3.14; N, 12.99%.

(*E*)-3-Amino-*N*'-(4-bromobenzylidene)-6-(4-nitrophenyl)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (14c) Orange solid (dioxane); m.p. above 300 °C; IR (v cm⁻¹): 3327, 3243 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 6.60 (br s, 2H, NH₂), 7.29 (t, 1H, thiophene-H4), 7.42 (d, 1H, thiophene-H5), 7.57 (d, 2H, Ar–H's), 7.79 (s, 1H, pyridine-H5), 7.84 (d, 2H, Ar–H's), 7.94 (d, 1H, thiophene-H3), 8.12 (s, 1H, methine-H), 8.17 (d, 2H, Ar–H's), 8.38 (d, 2H, Ar–H's), 11.56 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 94.2, 118.9, 121.9 (thienopyridine-*C*2, *C*5, *C*3a), 122.2, 123.8, 124.3, 127.7, 128.3, 128.7, 130.5, 131.3, 132.5, 136.4, 138.6 (Ar–*C*'s and thiophene-*C*'s), 141.6 (thienopyridine-*C*3), 143.2 (methine-*C*), 144.3 (Ar–*C*), 145.4, 146.2, 154.4 (thienopyridine-C4, C6, C7a), 168.2 (thienopyridine-CO); Anal. for $C_{25}H_{16}BrN_5O_3S_2$ (578.4): C, 51.91; H, 2.79; N, 12.11; found: C, 52.08; H, 2.67; N, 12.22%.

(E)-3-Amino-N'-(4-methylbenzylidene)-6-(4-nitrophenyl)-4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (14d) Yellow solid (dioxane); m.p. 294-297 °C; IR (v cm^{-1}): 3464, 3290, 3109 (NH, NH₂); ¹H-NMR (DMSO- d_6): δ 2.34 (s, 3H, CH₃), 6.68 (br s, 2H, NH₂), 7.31 (d, 2H, Ar-H's), 7.37 (t, 1H, thiophene-H4), 7.45 (d, 1H, thiophene-H5), 7.60 (d, 2H, Ar-H's), 7.79 (s, 1H, pyridine-H5), 7.92 (d, 1H, thiophene-H3), 8.08 (s, 1H, methine-H), 8.18 (d, 2H, Ar–H's), 8.37 (d, 2H, Ar–H's), 11.52 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): δ 19.8 (CH₃), 94.3, 119.6, 121.7 (thienopyridine-C2, C5, C3a), 122.4, 124.1, 127.3, 127.5, 128.8, 128.9, 130.6, 131.6, 136.6, 136.9, 138.7 (Ar-C's and thiophene-C's), 141.7 (thienopyridine-C3), 143.5 (methine-C), 144.4 (Ar-C), 145.1, 145.9, 154.3 (thienopyridine-C4, C6, C7a), 168.0 (thienopyridine-CO); Anal. for $C_{26}H_{19}N_5O_3S_2$ (513.5): C, 60.80; H, 3.73; N, 13.64; found: C, 60.68; H, 3.81; N, 13.47%.

(E)-3-Amino-N'-(4-hydroxybenzylidene)-6-(4-nitrophenyl) -4-(thiophen-2-yl)thieno[2,3-b]pyridine-2-carbohydrazide (14e) Orange solid (dioxane); m.p. above 300 °C; IR $(v \text{ cm}^{-1})$: 3443 (OH), 3338, 3244 (NH, NH₂); ¹H-NMR $(DMSO-d_6)$: δ 6.65 (br s, 2H, NH₂), 6.94 (d, 2H, Ar–H's), 7.36 (t, 1H, thiophene-H4), 7.47 (d, 1H, thiophene-H5), 7.63 (d, 2H, Ar-H's), 7.80 (s, 1H, pyridine-H5), 7.95 (d, 1H, thiophene-H3), 8.10 (s, 1H, methine-H), 8.18 (d, 2H, Ar-H's), 8.37 (d, 2H, Ar-H's), 10.05 (br s, 1H, OH), 11.50 (s, 1H, NH); ¹³C-NMR (DMSO- d_6): δ 95.2 (thienopyridine-C2), 115.6 (Ar-C), 117.9, 121.8 (thienopyridine-C5, C3a), 122.3, 124.1, 124.9, 127.8, 128.5, 128.7, 130.5, 136.6, 138.2 (Ar-C's and thiophene-C's), 140.3 (thienopyridine-C3), 144.2 (Ar-C), 144.6 (methine-C), 145.3, 145.6, 155.4 (thienopyridine-C4, C6, C7a), 159.5 (Ar-C), 167.9 (thienopyridine-CO); Anal. for C₂₅H₁₇N₅O₄S₂ (515.5): C, 58.24; H, 3.32; N, 13.58; found: C, 58.24; H, 3.32; N, 13.58%.

(*E*)-3-Amino-*N*'-(4-methoxybenzylidene)-6-(4-nitrophe nyl)-4-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carbohydrazide (14f) Yellow solid (dioxane); m.p. above 300 °C; IR ($v \text{ cm}^{-1}$): 3347, 3246 (NH, NH₂); ¹H-NMR (DMSOd₆): δ 3.82 (s, 3H, OCH₃), 6.68 (br s, 2H, NH₂), 7.08 (d, 2H, Ar–H's), 7.36 (t, 1H, thiophene-H4), 7.44 (d, 1H, thiophene-H5), 7.74 (d, 2H, Ar–H's), 7.83 (s, 1H, pyridine-H5), 7.93 (d, 1H, thiophene-H3), 8.08 (s, 1H, methine-H), 8.18 (d, 2H, Ar–H's), 8.39 (d, 2H, Ar–H's), 11.51 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): δ 55.3 (OCH₃), 94.5 (thienopyridine-C2), 114.4 (Ar–C), 118.6, 121.9 (thienopyridine-C5, C3a), 122.2, 123.9, 126.6, 127.8, 128.4, 128.7, 130.5, 136.6, 138.4 (Ar–C's and thiophene*C*'s), 140.8 (thienopyridine-*C*3), 144.1 (Ar–*C*), 144.3 (methine-*C*), 145.6, 146.3, 155.8 (thienopyridine-*C*4, *C*6, *C*7a), 160.6 (Ar–*C*), 167.7 (thienopyridine-*C*O); Anal. for $C_{26}H_{19}N_5O_4S_2$ (529.5): C, 58.97; H, 3.62; N, 13.22; found: C, 59.12; H, 3.48; N, 13.07%.

The in vitro antibacterial assay

The agar well diffusion method was used to evaluate the in vitro antibacterial activity of the new derivatives [42, 52] against each of Escherichia coli (ATCC:9637), Klebsiella pneumoniae (ATCC:10031), Staphylococcus aureus (ATCC:6538), Streptococcus mutans (ATCC:25175) and Enterococcus faecalis (ATCC:29212). The assay involved using of nutrient agar medium, Chloramphenicol (30 µg susceptibility disk, Unipath, Basingstoke, UK) as a reference drug and DMSO as a solvent and negative control. The hydrazones were tested using 15 mg/mL against each strain. Interpretation of results was done using of Chloramphenicol breakpoint for each strain. Petri dishes containing the sterilized media were prepared and allowed to solidify at rt. Microbial suspension in sterilized saline equivalent to McFarland 0.5 standard solution $(1.5 \times 10^5 \text{ CFU mL}^{-1})$ was prepared, whose turbidity modified to OD = 0.13 using spectrophotometer at 625 nm. In the solidified media, 6 mm diameter wells were made and 100 µL of each of the tested hydrazones was added. Then, the plates were left to stand for 24 h at 37 °C, and then, inhibition zones in mm were observed.

Minimum inhibitory concentration (MIC)

The twofold serial dilution method was used to evaluate the MIC values [53]. MIC value is the minimum concentration of the tested compound required to stop bacterial growth. For each strain of bacteria, 3-5 isolated colonies were transferred into 3-4 mL of sterile broth medium. The bacterial suspension was allowed to incubate for 2-6 h at 35-37 °C until its turbidity become equal to or greater than the turbidity of a McFarland Standard 0.5. Then, a stock solution of 1 mg/mL was prepared using DMSO as a solvent. The tested hydrazones were subjected to further progressive dilutions with the broth medium. Addition of a fixed volume of the prepared bacterial inoculum to each concentration of the tested hydrazones was done and then allowed to incubation for 16-20 h at 37 °C. The bacterial growth in the tested tubes was observed by comparing the resulted turbidity with the growth in the original inoculum without any of the tested hydrazones. For comparison, Chloramphenicol was tested in the same assay.

Cytotoxicity

Cell line, culture conditions and preparation of compounds

The human breast carcinoma cell line (MCF-7), human colon cancer cell line (Caco2) and human liver hepatocellular carcinoma cell line (HEPG2) were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

The selected cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM). All of the growth media were supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. Compounds **13a**, **13b**, **13c**, **13d**, **13g**, **13k**, **14b**, **14c** and **14d** as well as Doxorubicin, as a positive control, were dissolved in DMSO, and final concentrations were diluted in culture medium.

Neutral red uptake assay (NRU assay)

The NRU assay depends on the ability of living cells to bind neutral red, in lysosomes [54]. The cytotoxicity of hydrazones 13a, 13b, 13c, 13d, 13g, 13k, 14b, 14c and 14d was evaluated against each of MCF-7, Caco2 and HEPG2 cell lines using Doxorubicin as a standard drug. Exponentially growing cells were collected using 0.25% Trypsin–EDTA; then, the cell suspension was counted using hemocytometer, and cell viability was checked by trypan blue (100% viability). Then, the cells suspension was diluted with complete medium to have an approximately 1.0×10^5 cell/mL; then, 200 µL of the cell suspension was dispensed by multichannel pipette into the inner 60 wells of the 96 well plate, and the peripheral wells were filled with PBS; then, the plate incubated for 24 h before treatment with the tested compounds to allow attachment of cells to the wall of the plate. Different concentrations of the tested hydrazones (5, 25, 50 and 75 μ g/ mL) were prepared using DMEM media. 200 µL of treatment media was dispensed into 4 replicates for each concentration, and other wells were filled with untreated cells only (as a negative control) and wells filled with media containing Doxorubicin HCL as a positive control. The 96-well plate incubated at 37° C for 48 h. Then, the medium and extracts were discarded and replaced with 100 µL of neutral red solution (50 mg/mL) and centrifuged at 1800 rpm for ten min to remove any crystals of dye. After incubation at 37 °C for 3 h, the dye medium was discarded and the microplate was washed twice with 150 µL PBS to remove the unabsorbed neutral red dye contained in the wells. The cellular morphology of each of the treated MCF-7, Caco2 and HEPG2 cell lines with the tested hydrazones was observed using Inverted Microscope Leica DMI3000B. The absorbance of acidified ethanol solution containing extracted neutral red dye was

determined using microplate reader (BioTek, ELX808) at 540 nm to estimate the optical density, and the cell viability percentage was measured.

COX-2 inhibition assay

The ability of the tested derivatives to inhibit COX-2 enzyme was estimated [55, 56]. Briefly, COX-2 (purchased from Sigma-Aldrich) was added in 180 µL of the assay buffer containing 100 mM Tris-HCl buffer of pH 8.0, 1.0 mM hematin, 2.0 mM phenol and 5 mM EDTA. Each sample of the tested compounds and positive control, Celecoxib, was dissolved in DMSO and tested at three different concentrations (5, 10 and 25 µM). After addition of the tested derivatives or positive control (10 µL), the reaction mixture was allowed to incubate for 5 min at rt. The reaction was started by addition of 5 µL of arachidonic acid solution (5.0 mM) dissolved in methanol and N, N, N', N'-tetramethylpphenylenediamine dihydrochloride and allowed to incubate at 37 °C for 1 h. The reaction was quenched by adding 50 mL of 1 M HCl. The absorbance was measured using spectrophotometer at 610 nm. The inhibition percentage was estimated by comparing the test compounds with the blank and calculated using the equation:

 $([PGE2]_{vehicle} - [PGE2]_{drug}) \times 100/[PGE2]_{vehicle}$

All results were measured in triplicates. Compounds that inhibited 50% of COX-2 activity had its IC_{50} values calculated by using sigma plot software.

The Molecular docking: A tool of in silico studies

The in silico research was performed by study of molecular docking with COX-2 (PDB ID: 4cox) for some new hydrazones. In this study, a rigid framework for the Molecular Operating Environment (MOE) version 2019.01 was used [57]. MOE is one of the simple interactive molecular graphic tools used to test the feasible modes of docking of the set of ligands with COX-2 enzyme. The input of both the tested ligands and COX-2 must be in PDB format. The Gaussian 03 software was used to create a PDB file format of the structure of each ligand. The COX-2 structure was obtained from the web site of protein data bank (https://www.rcsb. org/). Only the amino acid chain is maintained, while other unsupported elements (e.g., Na, Mg, etc.) are detached [58].

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