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H₂O₂:HCl Catalyzed Synthesis of 5-(3-substituted-thiophene) Pyrimidine Derivatives and Evaluation for Their Pharmacological Effects

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Abstract

A series of 5-(3-substituted-thiophene) pyrimidine derivatives (**3a-d**) were synthesized *via* Knoevenagel condensation reaction in aqueous ethanol using H₂O₂:HCl as a green halogenating catalyst and evaluated for their pharmacological effects. The structures of the targets were confirmed by analytical and spectroscopic methods. From antibacterial activity result reveals that, all the four compounds showed appreciable activity with varied zone of inhibition, in that the compounds **3b** & **3d** exhibited most effective zone of inhibition against bacterial strains *E. coli* & *S. aureus* respectively. *In-vitro* cytotoxicity was carried by MTT assay method against MCF-7 (Breast cancer) cell line and results of all the four compounds showed excellent selectivity, in that compound **3a** exhibited excellent cytotoxicity with minimum cell viability range of 23.68 to 44.16 %. The interaction of compounds with CT-DNA was determined by using UV-absorption spectroscopy and results were confirmed that, all the synthesized compounds interacted strongly with CT-DNA through electrostatic or groove binding. *In-silico* ADME-toxicology results indicated that, all the targets are non-toxic, good oral bioavailability and druglikeness score indicated that they are suitable as drug-leads. From *In-silico* molecular docking results showed compound **3b** was bound with GlcN-6-P and P38 MAPk with least binding energy of -7.9 and -6.4 kcal/mol respectively.

Graphical Abstract

Keywords: H₂O₂: HCl catalyst, Antibacterial, Cytotoxicity, DNA binding, Molecular docking and ADME-toxicology study

Introduction

Heterocyclic compounds play a predominant role in medicinal chemistry and synthetic organic chemistry due to their massive biological importance. Sulphur and nitrogen containing heterocyclic compounds are always attempting to attract the attention of medicinal chemists and researchers due to their multiform pharmacological and biological activities [1-3]. Cancer is one of the leading diseases around the world and it caused by mutations in genes that regulate cell growth. The mutations led to abnormal cells division and multiply in an uncontrolled way. Breast cancer is the most common cancer in women worldwide, with more than 1.5 million new cases recorded every year. It is also the fifth highest cause of cancer death in world [4]. Current chemotherapies suffers major limitation of side effect and drug resistance, therefore continued search for novel and safer anticancer drugs remains as important [5]. Pyrimidine and thiophene have been recognized as key scaffolds due to its biological significances such as antiviral, antibacterial, antifungal, anticancer, anti-inflammatory, anti-diabetic, anti-tubercular, antioxidant, anti-parasitic, anti-convulsants, antidepressant, analgesic, antitumor, gastro protectors and kinase inhibitors [6, 7].

Moreover the heterocyclic compounds increase the strength of the complex by forming hydrogen bonds with DNA. Interactive study of heterocyclic moieties with DNA is essential for estimation of their anticancer activity and elucidates viable mechanism of their action. Hence, DNA binding is considered to be the most essential experimental step to measure the activity of anticancer drugs, because most of the anticancer drugs target DNA specifically [8].

Pollution is one of the critical problems faced by the synthetic organic chemist in designing the organic reactions for the synthesis of pharmacologically active compounds. Thus, the development of environment-friendly chemical process that induces necessary organic transformation is the important objectives of sustainable development [9]. Therefore, the “green chemistry” is an auspicious approach that meets the requirement of chemical and pharmaceutical industries. The replacement of hazardous solvents with eco-friendly solvents is an acceptable and valuable approach in a chemical reaction and use of a combination of hydrogen peroxide and the respective hydrohalic acid as a green halogenating agent [10]. Hypochlorous acid (HOCl or HClO) is a weak acid that forms when chlorine dissolves in water or H_2O_2 react with HCl and itself partially dissociates, forming hypochlorite (ClO^-). HOCl and ClO^- are powerful oxidizers and the primary disinfection agents [11, 12]. HOCl cannot be isolated from these solutions due to rapid equilibrium with its precursor. Earlier, our research group has reported different derivatives of pyrimidine moieties and other biologically important heterocyclic compounds [13, 14]. Some of the drugs containing pyrimidine nucleus available in the market (**Fig. 1**).

5-Fluorouracil
(Anticancer)

Floxuridine
(Anticancer)

Uramustine
(Anticancer)

Fig. 1 Some drugs containing pyrimidine nucleus

Based on above findings, here the application of $\text{H}_2\text{O}_2:\text{HCl}$ as a green halogenating catalyst for the synthesis of 5-(3-substituted-thiophene) pyrimidine derivatives as potent pharmacological agents.

Results and Discussion

Chemistry

In this report, we developed a simple and convenient method for the synthesis of 5-(3-substituted thiophene)-pyrimidine derivatives (**3a-d**) *via* Knoevenagel condensation of barbituric/thiobarbituric acid (**1**) with 3-substituted-thiophene-2-carboxaldehyde (**2**) in aqueous ethanol in the presence of $\text{H}_2\text{O}_2:\text{HCl}$ as a catalyst (**Scheme 1**).

Scheme 1 Synthesis of 5-(3-substituted-thiophene) pyrimidine derivatives (**3a-d**)

A possible mechanism for the formation of 5-(3-substituted-thiophene) pyrimidine derivatives has been shown in **Scheme 2**. Firstly, hypochlorous acid (HOCl) was formed by the reaction of H₂O₂ with HCl and it was act as powerful oxidising agent and then the reaction was initiated by the generation of carbanion **2** from active methylene compound **1**. The carbanion **2** attacks on the carbonyl carbon of aldehyde **3** to form intermediate **4** which undergo subsequent dehydration to desired Knoevenagel product **5**. The abstraction of acidic proton from active methylene compound and electrophilicity of carbonyl group of aldehydes are increased by HOCl due to hydrogen bonding.

Scheme 2 Possible mechanism of synthesized compounds (3a-d)

Firstly, we studied the effect of catalyst on the reaction. In the previous reports, the same reaction was carried out in presence of different catalysts such as CuO NPs, PVP-Ni NPs, Fe₃O₄ NPs, L-tyrosine, NH₂SO₃H, EAN, Bi(NO₃)₃. 5H₂O and also in the absence of catalyst (**Table 1**), were used to find the influence of catalyst in progress of reaction as well as in the increase of product yield. But our observation of the experiment revealed that, the starting materials remained in the reaction media which was clearly indicated in the TLC even prolonged time of stirring. Therefore we concluded that the best result was obtained in the presence green halogenating catalyst H₂O₂:HCl, whereas further increase in the quantity of catalyst doesn't have a significant effect on reaction kinetics.

Table 1 Effect of catalyst on synthesized compound **3a**

Entry	Catalyst	Solvent	Temperature (°C)	Time (min)	Yield (%)
1	H ₂ O ₂ : HCl	EtOH: H ₂ O	Reflux	10	96
2	CuO NPs	-	RT	10	93 ^[15]
3	PVP-Ni NPs	Ethylene glycol	Reflux	10	87 ^[16]
4	Fe ₃ O ₄ NPs	EtOH	Reflux	30	70 ^[17]
5	L-tyrosine	H ₂ O	RT	16	93 ^[18]
6	NH ₂ SO ₃ H	-	Grinding	120	96 ^[19]
7	EAN	Ionic liquids	RT	10	96 ^[20]
8	Bi(NO ₃) ₃ .5H ₂ O	EtOH	Reflux	20	95 ^[21]
9	-	EtOH	Reflux	120	89 ^[22]

Consequently, to study the effect of temperature on synthesized compound **3a**, we carried a reaction at RT, 50 °C and 80 °C (**Table 2**) as a result while increase the reaction temperature decreases the reaction time from 60 to 20 min and 20 to 10 min respectively, but yield of the product does not affected by increase in temperature.

Table 2 Effect of temperature on synthesized compound **3a**

Entry	Temperature (°C)	Time (min)	Yield (%)
1	RT	60	96
2	50	20	96
3	80	10	96

The structures of the intended 5-(3-substituted-thiophene) pyrimidine derivatives (**3a-d**) were confirmed by IR, ^1H NMR, ^{13}C NMR and HRMS spectral data. IR spectrum of compound **3a** showed the absorption band in the region 3373 cm^{-1} is attributed to the amide stretching vibration and the absorption band at 1654 cm^{-1} corresponds to stretching vibration of the carbonyl group (C=O). Another stretching vibrational band at 1529 cm^{-1} corresponds to C=C bond. The ^1H NMR spectrum of compound **3a** exhibited two singlet peaks at δ 11.21 and 11.17 ppm which corresponds to two NH protons of pyrimidine nucleus (s, 2H, NH) and another singlet peak at δ 8.53 ppm due to CH proton (s, 1H, CH). A multiplet peak was observed in the range of δ 8.24-8.13 ppm corresponds to two aromatic protons (m, 2H, Ar-H) and a triplet peak at 7.33-7.31 ppm due to one aromatic proton (t, J = 8 Hz, 1H, Ar-H). In addition, ^{13}C NMR spectrum of the compound **3a** exhibited peaks at δ 163.950 and 163.453 ppm which correspond to carbonyl carbons.

The mass spectrum showed molecular ion peak $[\text{M}+\text{H}]^+$ at m/z is 221. 9054 which correspond to the molecular weight of the compound **3a** (Supporting information: S1 to S16). The physical and analytical data of synthesized compounds (**3a-d**) was appended in **Table 3**.

Table 3 Physical and analytical data of synthesized compound (**3a-d**)

Entry	Comp.	X	R	Product	Yield (%)	MP ($^{\circ}\text{C}$)	
						Observed	Reported
1	3a	O	H		98	278-280	271
2	3b	O	CH_3		95	302-306	-

3	3c	S	H		94	320-322	>300
4	3d	S	CH ₃		96	310-312	-

Pharmacological effect

Antibacterial activity

The synthesized 5-(3-substituted-thiophene) pyrimidine derivatives (**3a-d**) were screened for their *in-vitro* antibacterial activity at two different concentrations (20 & 40 µg/mL) as shown in **Fig. 2**. All the four compounds showed appreciable antibacterial activity with varied zone of inhibition in the range of 3.3 to 3.8 & 7.1 to 7.8 mm against *E. coli* and 3.0 to 3.7 & 7.4 to 7.9 mm against *S. aureus* respectively, the results were appeared in **Table 4**. Also results revealed that, the compounds **3b** (3.8 and 7.8 mm) and **3d** (3.7 and 7.9 mm) having electron donating group (methyl) on C-3 of the thiophene ring were exhibited most inhibitory effect against bacterial strains *E. coli* & *S. aureus* respectively as compared with standard drug Ciproflaxin.

Table 4 Antibacterial activity results of synthesized compounds (**3a-d**)

Compd.	Zone of inhibition in mm			
	<i>Escherichia coli</i>		<i>Staphylococcus aureus</i>	
	Concentration in µg/mL			
	20	40	20	40
3a	3.5	7.8	3.0	7.5
3b	3.8	7.8	3.1	7.6
3c	3.3	7.2	3.5	7.4
3d	3.4	7.1	3.7	7.9
Ciproflaxin	4	8	4.2	8.4

Fig. 2 Images of antibacterial study of the synthesized compounds (**3a-d**)

Cytotoxicity study

All the four synthesized compounds were investigated for their *in-vitro* cytotoxicity against MCF-7 (Breast cancer) cell line (**Fig. 3**). Plot of compound concentration versus the survival fraction was performed (**Fig. 4**) and percentages of cell survival of the tested compounds are listed in **Table 5**.

From *In-vitro* cytotoxicity results revealed that, all the four compounds displayed an outstanding selectivity against MCF-7 cell line. Among the compounds, compound **3a** exhibited excellent cytotoxicity with minimum cell survival range of 23.68 to 44.16 % at the concentration range of 200 to 6.25 $\mu\text{g/mL}$ respectively. Whereas 3b, 3c & 3d were also

displayed reliable selectivity at all concentrations with cell survival ranges of 29.00 to 50.93 %, 31.31 to 66.82 % and 26.95 to 53.12 % respectively.

Table 5 Percentage of cell viability against MCF-7 cell line of the synthesized compounds (3a-d)

Concentration in $\mu\text{g/mL}$	Mean cell Viability of MCF-7			
	3a	3b	3c	3d
6.25	44.16 $\pm$ 0.76	50.93 $\pm$ 0.42	66.82 $\pm$ 0.41	53.12 $\pm$ 0.34
12.5	38.11 $\pm$ 0.82	48.68 $\pm$ 0.31	58.1 $\pm$ 0.13	46.88 $\pm$ 0.52
25	34.09 $\pm$ 1.2	43.72 $\pm$ 0.52	48.6 $\pm$ 0.23	43.93 $\pm$ 0.42
50	32.37 $\pm$ 0.82	40.23 $\pm$ 0.61	40.03 $\pm$ 0.82	40.03 $\pm$ 0.62
100	30.06 $\pm$ 0.62	36.8 $\pm$ 0.20	37.38 $\pm$ 0.61	37.85 $\pm$ 0.34
200	23.68 $\pm$ 0.41	29 $\pm$ 0.16	31.31 $\pm$ 0.42	26.95 $\pm$ 0.52

Values are Mean $\pm$ SE, N=3, *P<0.01 vs. Control

Fig. 3 Images of anticancer study of the synthesized compounds (**3a-d**)

Fig. 4 A graph of % of surviving cells of compounds (**3a-d**) at different concentration against MCF-7 cell line

DNA binding study

DNA binding study was assessed by using electronic spectroscopy. The UV-absorption spectral studies were employed to examine the binding mode of compounds to CT-DNA, involves the changes in absorbance and wavelength [23]. The molecules are bind to DNA

with two modes (covalent or non-covalent of binding). Covalent bonding led to bathochromism and hyperchromism due to breaking of the DNA structure when a compound interacted with DNA covalently. While in non-covalent binding there are “electrostatic”, “groove” and “intercalative” types of interactions. Decreased in absorption (hypochromic shifts) and red shift (bathochromic shift) revealed the intercalative binding of compounds with DNA. Lower hypochromic/hyperchromic effect with no or negligible bathochromic shift led to electrostatic binding. Minor or no effect and rarely, some hyperchromism shows the groove binding [24-26].

The DNA binding efficiency of synthesized compounds (**3a-d**) was monitored by comparing their absorption spectra with and without CT-DNA. The absorption spectra was carried out at fixed concentration of synthesized compounds and varying with DNA concentrations (25-350 μL of 0.5025×10^{-7} to $6.0670 \times 10^{-7} \text{ molL}^{-1}$) under physiological condition of pH 7.01. The absorption spectra of all the synthesized compounds (**3a-d**) exhibited absorption bands at 235 to 240 nm due to $\pi\text{-}\pi^*$ transitions, the resultant spectral graphs are shown in **Fig. 5**. The K_b values of compounds (**3a-d**) are found to be 1.1216×10^7 , 1.4072×10^7 , 1.0634×10^7 and 3.4872×10^7 respectively appear in **Table 6**. These K_b values conforms all the synthesized compounds interacted strongly with CT-DNA. Among the four compounds, compound **3d** showed a prominent binding ability with CT-DNA compare to other compounds. The absorption bands of the compounds were affected due to the gradual increasing of CT-DNA concentration resulting hyperchromism/hypochromism, no/or negligible blue/red shift indicates strong interaction of the compounds with CT-DNA mainly through electrostatic or groove binding [27]. The kinetics and thermodynamics of compounds-DNA interaction in terms of binding constant (K_b) and Gibbs free energy change (ΔG) were evaluated by using the classical Van't Hoff's equation, $\Delta G = -2.303RT \log K_b$. The negative ΔG values confirmed spontaneous binding of compounds with CT-DNA through formation of stable complexes.

Table 6 DNA binding results of synthesized compounds (**3a-d**)

Compd.	$\lambda_{\max}$ (nm)		$\Delta\lambda_{\max}$ (nm)	% H	K_b (M ⁻¹)	ΔG (kJ/mol)
	Free	Bound				
3a	240	239	1	6.1879×10^{-4}	1.1216×10^7	-40.205
3b	236	236	0	6.0157×10^{-4}	1.4072×10^7	-40.767
3c	239	239	0	1.1560×10^{-3}	1.0634×10^7	-40.073
3d	236	236	0	1.2388×10^{-3}	3.4872×10^7	-43.015

Fig. 5 The electronic absorption spectra of compounds (**3a-d**) in the absence and presence of increasing amounts of CT-DNA. Arrow (↓) shows the change in the absorbance with increase the DNA concentration. Inset: plot of $[DNA]/(\epsilon_a - \epsilon_f)$ Vs $[DNA]$.

***In-silico* ADME-toxicology studies**

The bioavailability and druglikeness were estimated for all the synthesized compounds (**3a-d**) based on the molecular properties and the results indicated that, all the four compounds under study passes through Lipinski's filter without any violation, further demonstrating a positive druglikeness score indicating their suitability as drug-leads. *In-silico* pharmacokinetic studies indicated that, all the molecules under study can penetrate blood brain barrier and readily absorbed by human intestine while they are impermeable to Caco-2; an immortalized cell line of human colorectal adenocarcinoma cells and non-substrate to Cytochromes P450 (CYP450) group of enzymes (**Table 7**).

In-silico pharmacodynamics studies revealed that, all the four molecules are non-mutagenic, non-tumorigenic, non-irritant, AMES non-toxic with high reproductive effects and possible hepatotoxicity. The bioactivity assessment indicated that, the molecules do not belong to GPCR group of ligands, do not modulate ion channels, non-kinase inhibitors, non-nuclear receptor ligands, non-protease and non-enzyme inhibitors (**Table 8**).

Table 7 Bioavailability, druglikeness and *in-silico* pharmacokinetic assessment of synthesized compounds (**3a-d**)

Comp.	Bioavailability and Druglikeness							<i>In-silico</i> Pharmacokinetics			
	Total Molecular weight	cLogP	H-Acceptors	H-Donors	Rotatable Bonds	Polar Surface Area	Druglikeness	Human intestinal absorption	Caco-2 permeability	Blood brain barrier	CYP450 2D6 substrate
3a	222.224	0.2739	5	2	1	103.51	5.2698	+0.982	-0.779	+0.982	-0.873
3b	236.251	0.6178	5	2	1	103.51	4.9763	+0.728	-0.753	+0.980	-0.889
3c	238.291	0.6344	4	2	1	118.53	4.2229	+0.979	-0.698	+0.977	-0.871
3d	252.318	0.9783	4	2	1	118.53	3.9143	+0.987	-0.673	+0.976	-0.886

Table 8 *In-silico* pharmacodynamics and bioactivity assessment of synthesized compounds (**3a-d**)

Comp.	<i>In-silico</i> Pharmacodynamics							Bioactivity score					
	Mutagenic	Tumorigenic	Reproductive Effective	Irritant	Aerobic biodegradability	Ames toxicity	Hepatotoxicity	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
3a	NONE	NONE	HIGH	NONE	-0.590	-0.706	+0.925	-1.12	-1.58	-0.86	-1.26	-1.30	-0.69
3b	NONE	NONE	HIGH	NONE	-0.562	-0.694	+0.950	-1.07	-1.44	-0.90	-0.91	-1.32	-0.73
3c	HIGH	NONE	HIGH	NONE	-0.859	-0.731	+0.900	-1.43	-1.77	-1.41	-1.73	-1.32	-1.00
3d	HIGH	NONE	HIGH	NONE	-0.843	-0.711	+0.850	-1.35	-1.62	-1.42	-1.35	-1.34	-1.03

***In-silico* molecular docking studies**

The *in-silico* molecular docking studies were performed to predict the most effective binding among the synthesized molecules to appropriate targets [28, 29]. The results revealed that, the compound **3b** was bound with GlcN-6-P and P38 MAPk with minimum binding energy of -7.9 and -6.4 kcal/mol while the molecules **3a**, **3d** and **3c** interacted with a binding energy of -7.6 and -6.4, -7.4 and -6.2 and -7.4 and -6.0 with GlcN-6-P and P38 MAPk targets respectively. The interaction of all the molecules with GlcN-6-P and P38 MAPk were compared with antibacterial agent Ciprofloxacin (-7.7 kcal/mol) and anticancer agent 5-fluorouracil (-4.7 kcal/mol) and results are appended in **Table 9**.

Table 9 Binding energies of synthesized compounds (**3a-d**) and standard drugs with GlcN-6-P and P38 MAPk targets

Antibacterial activity		Anticancer activity	
Compd.	Binding energy in kcal/mol	Compd.	Binding energy in kcal/mol
Ciprofloxacin	-7.7	5-Fluorouracil	-4.7
3a	-7.6	3a	-6.4
3b	-7.9	3b	-6.4
3c	-7.4	3c	-6.0
3d	-7.4	3d	-6.2

Fig. 6 Binding interaction of compounds **3a** (a&b), **3b** (c&d), **3c** (e&f) and **3d** (g&h) with GlcN-6-P along with reference standard ciprofloxacin (i&j) (a) & Binding interaction of compounds **3a** (a&b), **3b** (c&d), **3c** (e&f) and **3d** (g&h) with P38 MAPK along with reference standard 5-fluorouracil (i&j) (b)

Conclusion

We have described mild, easy and green protocol for the synthesis of 5-(3-substituted-thiophene) pyrimidine derivatives (**3a-d**) using H₂O₂:HCl as a catalyst under reflux condition. This synthetic approach has short reaction time, excellent yield and clean reactions make this procedure magnificent alternative to the existing methods. Furthermore, this method is environmentally greener and safer method. The activity results revealed that, the compounds **3b** & **3d** exhibited more potent antibacterial activity against *E. coli* & *S. aureus* respectively compared to standard drug Ciproflaxin. *In-vitro* cytotoxicity results disclosed that, all the synthesized compounds showed outstanding selectivity on MCF-7 cell line, in that compound **3a** exhibited most effective cytotoxicity with minimum cell survival range of 23.68 to 44.16 %. DNA binding results clearly indicates that, all the synthesized compounds interacted strongly with CT-DNA and compound-DNA complexes were stabilized by electrostatic or groove binding. *In-silico* ADME-toxicology results indicated that, all the four compounds are non-toxic and good oral bioavailability and druglikeness score indicated that they are suitable as drug-leads. From *In-silico* molecular docking results, the compound **3b** was bound with GlcN-6-P and P38 MAPk with least binding energy of -7.9 and -6.4 kcal/mol respectively. In future, the obtained compounds can be used as antibiotics, anticancer agents, dyes and pigments, paints and food industries.

Experimental

Materials and Method

All chemicals and calf thymus DNA were purchased from Aldrich Chemical Company and reaction was performed at refluxed condition and solvents were used without further purification. Analytical TLC was performed with E. Merck silica gel GF254 glass plates. Visualization of the developed chromatogram was performed by UV light (254 and 356 nm). The melting points of the products were determined in open capillary tubes and uncorrected.

The ATR-IR spectra were obtained using Bruker FTIR Alpha spectrometer. The ^1H NMR and ^{13}C NMR spectra were recorded on Bruker 400 MHz and 100 MHz in DMSO- d_6 as a solvent respectively. Mass spectra were obtained by Agilent 1200 series LC & Micromass Q spectrometer. DNA binding studies were carried on Elico SL 159 UV-Visible spectrophotometer in 200-500 nm range equipped with 1.0 cm quartz cell at room temperature. The anticancer activity was carried out in the Dept. of Microbiology, Maratha Mandal's NGH Institute of Dental Sciences & Research Centre, Belgaum, Karnataka.

General procedure for the synthesis of 5-(3-substituted-thiophene) pyrimidine derivatives (3a-d)

A mixture of barbituric/thiobarbituric acid (**1**, 1mmol) with 3-substituted-thiophene-2-carboxaldehyde (**2**, 1mmol) in 15 mL aqueous ethanol using $\text{H}_2\text{O}_2:\text{HCl}$ (2:1) as a catalyst and refluxed with constant stirring for about 10-15 min. simultaneously, the reaction was monitored by TLC (Ethyl acetate & Petroleum ether). After completion of reaction, the reaction mixture was cooled to room temperature and poured into the 100 mL flake ice with vigorous stirring to get solid precipitated out, filtered, washed with absolute ethanol and dried to afford pure solid products (**3a-d**).

5-(Thiophen-2-ylmethylidene)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (3a): Yellow solid, yield-96%; mp 280-282 °C; IR (ATR, ν cm^{-1}): 3373 (NH), 1654 (C=O), 1529 (C=C); ^1H NMR (400 MHz, DMSO- d_6 , δ ppm): 7.31-7.33 (t, J = 8 Hz, 1H, Ar-H), 8.13-8.24 (m, 2H, Ar-H), 8.53 (s, 1H, CH), 11.17 (s, 1H, NH), 11.21 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm): 112.035, 128.808, 136.776, 142.526, 146.116, 146.291, 150.679, 163.453 and 163.950 (C=O); HRMS: m/z 221.9054 $[\text{M}+\text{H}]^+$. Anal. Calcd for $\text{C}_9\text{H}_6\text{N}_2\text{O}_3\text{S}$: C, 48.64; H, 2.72; N, 12.61. Found: C, 48.59; H, 2.68; N, 12.53.

5-[(3-Methylthiophen-2-yl)methylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (3b): Yellow solid, yield-94%; mp 302-306 °C; IR (ATR, ν cm⁻¹): 3225 (NH), 2858 (CH₃), 1703 (C=O), 1542 (C=C); ¹H NMR (400 MHz, DMSO-d₆, δ ppm): 2.24 (s, 3H, CH₃), 7.21-7.23 (d, *J*= 8 Hz, 1H, Ar-H), 8.16-8.18 (d, *J*= 8 Hz, 1H, Ar-H), 8.53 (s, 1H, CH), 11.17 (s, 1H, NH), 11.24 (s, 1H, NH); ¹³C NMR (100 MHz, DMSO-d₆, δ ppm): 19.922 (CH₃), 114.870, 135.252, 136.011, 145.166, 147.180, 154.915, 158.106, 167.728 and 168.497 (C=O); HRMS: *m/z* 235.9774 [M+H]⁺. Anal. Calcd for C₁₀H₈N₂O₃S: C, 50.84; H, 3.41; N, 11.86. Found: C, 50.79; H, 3.36; N, 11.80.

5-(Thiophen-2-ylmethylidene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (3c): Green solid, yield-93%; mp 320-322 °C; IR (ATR, ν cm⁻¹): 3106 (NH), 1760 (C=O), 1559 (C=C), 1156 (C=S); ¹H NMR (400 MHz, DMSO-d₆, δ ppm): 6.86-6.88 (d, *J*= 8 Hz, 1H, Ar-H), 8.20 (s, 1H, CH), 8.34-8.36 (d, *J*= 8 Hz, 2H, Ar-H), 12.20 (s, 1H, NH), 12.30 (s, 1H, NH); ¹³C NMR (100 MHz, DMSO-d₆, δ ppm): 114.713, 116.139, 124.434, 139.254, 157.012, 160.480, 162.825, 164.168 (C=O) and 178.617 (C=S); HRMS: *m/z* 237.9467 [M+H]⁺. Anal. Calcd for C₉H₆N₂O₂S₂: C, 45.36; H, 2.54; N, 11.76. Found: C, 45.30; H, 2.49; N, 11.71.

5-[(3-Methylthiophen-2-yl)methylidene]-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (3d): Green solid, yield-95%; mp 310-312 °C; IR (ATR, ν cm⁻¹): 3108 (NH), 2852 (CH₃), 1704 (C=O), 1516 (C=C), 1200 (C=S); ¹H NMR (400 MHz, DMSO-d₆, δ ppm): 2.24 (s, 3H, CH₃), 7.22-7.24 (d, *J*= 8 Hz, 1H, Ar-H), 8.17-8.19 (d, *J*= 8 Hz, 1H, Ar-H), 8.54 (s, 1H, CH), 11.18 (s, 1H, NH), 11.25 (s, 1H, NH); ¹³C NMR (100 MHz, DMSO-d₆, δ ppm): 26.806 (CH₃), 114.280, 128.031, 128.903, 129.867, 130.391, 130.892, 142.472, 163.552 (C=O) and 173.358 (C=S); HRMS: *m/z* 251.9608 [M+H]⁺. Anal. Calcd for C₁₀H₈N₂O₂S₂: C, 47.60; H, 3.20; N, 11.10. Found: C, 47.55; H, 3.16; N, 11.06.

Pharmacological studies

Antibacterial activity

Antibacterial activity of the synthesized compounds (**3a-d**) was carried out by agar well diffusion method [30] using two bacterial strains *Escherichia coli* (MTCC-1559) and *Staphylococcus aureus* (MTCC-902). DMSO was used as negative control and Ciprofloxacin as standard drug. The test compounds were dissolved in DMSO at two different concentrations 20 & 40 µg/mL.

Cytotoxicity

In-vitro cytotoxicity was assessed by MTT assay method [31] against MCF-7 (Breast cancer) cell line. The cells were seeded a 96-well flat-bottom micro plate and maintained at 37 °C in 95% humidity and 5% CO₂ for overnight. Different concentration (200, 100, 50, 25, 12.5 and 6.25 µg/mL) of samples were treated. The cells were incubated for another 48 hours and the wells were washed twice with PBS and 20 µL of the MTT staining solution was added to each well and plate was incubated at 37 °C. After 4h, 100 µL of DMSO was added to each well to dissolve the formazan crystals and absorbance was recorded with a 570 nm using micro plate reader. The percentage of cell survival was calculated by using following formula.

$$\% \text{ of cell survival} = \frac{\text{Mean OD of test compound}}{\text{Mean OD of Negative control}} \times 100$$

DNA binding study

DNA binding study was assessed by using electronic spectroscopy. A solution of CT-DNA in 50 mM Tris-HCl/50 mM NaCl buffer solution was prepared at pH 6.9-7.01. In buffer solution, the ratio of absorption values of CT-DNA at 260 and 280 nm is 1.8-1.9, indicates that DNA was free of proteins [32]. Then a concentrated stock solution of DNA was prepared in 50 mM Tris HCl/50 mM NaCl in double distilled water at pH 6.9-7.01 and the concentration of CT-DNA was determined per nucleotide by taking the absorption coefficient

(6600 dm³mol⁻¹cm⁻¹) at 260 nm [33]. Stock solutions were stored at 4 °C and it was used before 4 days. A 2 mL solution in 1cm quartz containing fixed concentration of the compounds (**3a-d**) with calf thymus DNA (0 to 350 µL of a 0.5025-6.0670x10⁻⁷ M stock CT-DNA solution). A blank solution containing the same concentration of DNA was used as a reference. Solutions were prepared by mixing the compound and CT-DNA in DMSO medium, and then record the UV absorption spectra by addition of 25 to 350 µL DNA to the compound. The spectra were recorded against blank solution containing same concentration of DNA (4.0909x10⁻⁶ molL⁻¹). Then the intrinsic binding constant K_b can be obtained by following equation [34].

$$\frac{[\text{DNA}]}{(\epsilon_A - \epsilon_B)} = \frac{[\text{DNA}]}{(\epsilon_B - \epsilon_F)} + \frac{1}{K_b(\epsilon_B - \epsilon_F)} \quad (1)$$

Where, ϵ_A, ϵ_B and ϵ_F corresponds to the apparent, bound and free compound extinction coefficients respectively. A plot of $\frac{[\text{DNA}]}{(\epsilon_A - \epsilon_F)}$ versus $[\text{DNA}]$ gave a slope of $\frac{1}{(\epsilon_B - \epsilon_F)}$ and Y-intercept equal to $\frac{1}{K_b(\epsilon_B - \epsilon_F)}$. Hence K_b is the ratio of slope to intercept. The % of hyperchromicity or hypochromicity (% H) for the CT-DNA/[Ligand] was obtained from $(\epsilon_a - \epsilon_f) / \epsilon_f \times 100$.

***In-silico* oral bioavailability assessment and ADME-toxicology studies**

The oral bioavailability of the synthetic molecules (**3a-d**) can be predicted by considering their structural properties to screen based on the Rule of five or Lipinski rule-of-five (RO5) filter [35]. Rule of five describes the molecular properties necessary to filter candidate drug's pharmacokinetic (PK) and pharmacodynamics (PD) [36-38].

Oral bioavailability assessment was done using Osiris Data warrior V.4.4.3 [39] based on total molecular weight, ClogP, H-acceptors, H-donors, rotatable bonds as part of RO5 filters, along with TPSA (Topological polar surface area) and druglikeness assessment [40].

Pharmacodynamic properties like mutagenicity, tumorigenicity, reproductive effective, irritant properties, Ames toxicity and hepatotoxicity were predicted using admetSAR [41] server. Bioactivity scores were predicted using molinspiration [37] for GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor inhibitor, protease inhibitor, enzyme inhibitor. Pharmacokinetic properties like blood-brain barrier penetration, human intestinal absorption, Caco-2 permeability and CYP450 2D6 substrate were predicted by submitting each molecule individually to admetSAR server [41, 42].

***In-silico* molecular docking studies**

The docking of the synthesized compounds to the binding pocket of glucosamine-6-phosphate synthase (GlcN-6-P) and P38 MAP kinase was carried out using Autodock Vina program [43]. The co-crystallized structure of GlcN-6-P (PDB ID: 2VF5) and P38 MAP kinase (PDB ID: 1OUK) were retrieved from protein databank and their substrate binding sites were identified using pdbsum server [42, 44, 45]. A grid box of dimensions 40 x 50 x 40 Å with X, Y & Z coordinates at 32.198, 16.709 and -3.151 for GlcN-6-P and 56 x 60 x 48 Å with X, Y & Z coordinates at 44.746, 34.234 and 32.603 for P38 MAPk were created respectively. For the obtained molecules, all the torsions were allowed to rotate during docking [38]. The grid box was set around the residues forming the active pocket [40]. The binding interactions were visualized using Biovia Discovery Studio Visualizer V.20.1 and Schrodinger-Maestro V.12.7. The *in-silico* studies were performed on a local machine equipped with AMD Ryzen 5 six-core 3.4 GHz processor, 8GB graphics and 16 GB RAM with Microsoft Windows 10 operating system.

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Supporting information:

Spectroscopic spectra's (IR, ^1H NMR, ^{13}C NMR and Mass spectrometry) of the synthesized compounds have been provided in the supporting information file.

Conflict of Interest: The authors declare that there is no conflict of interests regarding the publication of this work.

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H₂O₂:HCl Catalyzed Synthesis of 5-(3-substituted-thiophene) Pyrimidine

Derivatives and Evaluation for Their Pharmacological Effects

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Graphical representation

Figures

Figure 1

Some drugs containing pyrimidine nucleus

Figure 2

Images of antibacterial study of the synthesized compounds (3a-d)

Figure 3

Images of anticancer study of the synthesized compounds (3a-d)

Figure 4

A graph of % of surviving cells of compounds (3a-d) at different concentration against MCF-7 cell line

Figure 5

The electronic absorption spectra of compounds (**3a-d**) in the absence and presence of increasing amounts of CT-DNA. Arrow (X) shows the change in the absorbance with increase the DNA concentration. Inset: plot of $[DNA]/(\epsilon a - \epsilon_f)$ Vs $[DNA]$.

Figure 6

Binding interaction of compounds 3a (a&b), 3b (c&d), 3c (e&f), and 3d (g&h) with GlcN-6-P along with standard drug ciprofloxacin (i&j) (a) & Binding interaction of compounds 3a (a&b), 3b (c&d), 3c (e&f), and 3d (g&h) with P38 MAPk along with standard drug 5-fluorouracil (i&j) (b)

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