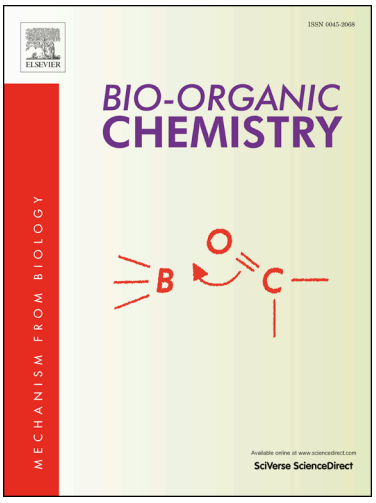

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PII: S0045-2068(18)30733-8
DOI: https://doi.org/10.1016/j.bioorg.2018.09.022
Reference: YBIOO 2519

To appear in: *Bioorganic Chemistry*

Received Date: 24 July 2018
Revised Date: 10 September 2018
Accepted Date: 11 September 2018

Please cite this article as: M. M.Fouad, E.R. El-Bendary, G.M. suddek, I.A. Shehata, M. M.El-Kerdawy, Synthesis and *in vitro* antitumor evaluation of some new thiophenes and thieno[2,3-d]pyrimidine derivatives, *Bioorganic Chemistry* (2018), doi: https://doi.org/10.1016/j.bioorg.2018.09.022

Synthesis and *in vitro* antitumor evaluation of some new thiophenes and thieno[2,3-d]pyrimidine derivatives

Mahasen M.Fouad[a*], Eman R. El-Bendary[a,b], Ghada M. suddek[c], Ihsan A. Shehata[a], Mohamed M.El-Kerdawy[a]

[a]Department of Medicinal Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.
[b] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Horus University, Egypt.
[c]Department of Pharmacology, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.

[*]Corresponding author ; *E-mail* address: sonas_sonas_91@yahoo.com (Mahasen M. Fouad) Tel: +20502367574

Keywords: Thiophenes, Thienopyrimidines, Synthesis, Antitumor evaluation.

Abstract

New thiophene(**2-13**) and thienopyrimidine (**15-27**) derivatives have been synthesized. Twenty three compounds were screened against five cell lines namely; hepatocellular carcinoma (liver) HepG-2, epidermoid carcinoma (larynx) Hep-2, mammary gland (breast) MCF-7, human prostate cancer PC-3 and epitheloid cervix carcinoma HeLa. The results revealed that compounds **15**,**16**,**17**,**24** and **25** showed the highest antitumor activity against all tested cell lines compared to Doxorubicin. In order to explain the expected mode of action of the observed anticancer activity, compounds **15**,**16**,**17**,**24** and **25** were selected to screen their DNA binding affinity and enzyme inhibitory activity against DNA polymerase, thymidylate synthase and tyrosine kinase. The results revealed that the tested compounds showed good DNA binding affinity as well as good inhibitory activity against the three enzymes which might explain the observed anticancer activity of the target compounds.

1. Introduction

Cancer is defined as large groups of diseases in which cells abnormally divide invading the body tissues and organs and eventually spreading throughout the body[1].Cancer is a fatal disease caused over 8.7million deaths globally in 2015 taking the second place after cardiovascular diseases as leading causes of death[2].

Till now there is no practical and completely effective drugs or ways to control the unstopping progress of this disease. Therefore, there is a continuous global efforts to identify novel effective, selective and less toxic anticancer agents[3, 4].

Thiophenes have been reported to have many types of biological activities, where many of them used as theraputic agents as antimicrobial[5-7], anticancer[8-10],anti-inflammatory[11] and antiviral agents[12].Also, it was reported that thienopyrimidines have promising anticancer activity[13-16] with different mechanisms as it may act as inhibitors for thymidylate synthase [17] and protien kinase [18-22].Moreover, compounds containing morpholine and piperazine moieties have been reported to posses good anticancer activity[23].

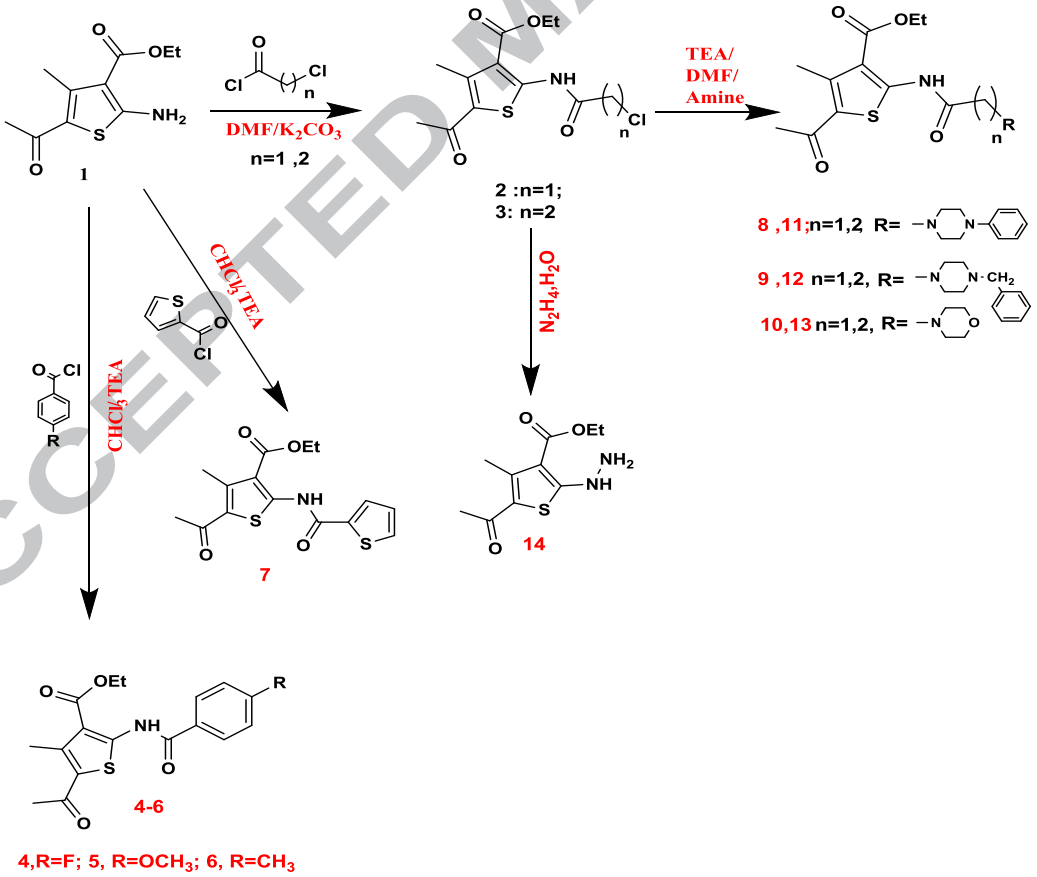
In the light of the above considerations, we synthesized new thiophene derivatives bearing morpholine and piperazine moieties in addition to new thieno[2,3-d]pyrimidin-4(3H)-one derivatives. The new compounds were evaluated for anticancer activity. DNA-binding affinity and enzyme inhibitory action were also evaluated on the active derivatives in order to inspect their preliminary mode of action.

2.Results and discussion

2.1. Chemistry

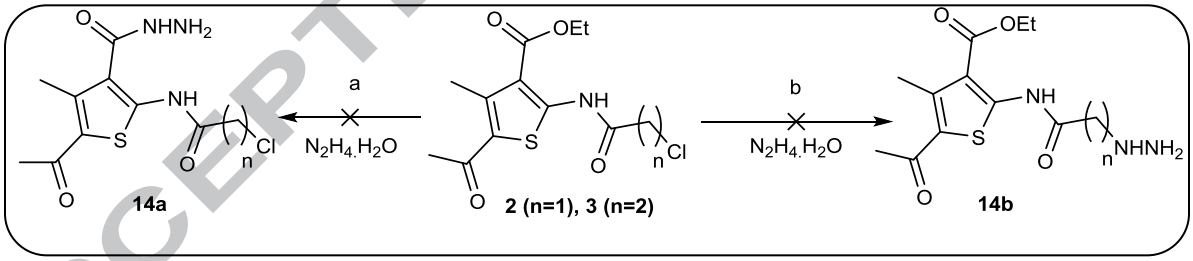
Synthesis of ethyl 5-acetyl-2-amino-4-methylthiophene-3-carboxylate **1** was prepared *via* multicomponent condensation between acetyl acetone, ethyl cyanoacetate, sulfur and diethyl amine in absolute ethanol according to reported procedure[24].The N-acylation of amino group of compound **1** by using chloroacetyl chloride and chloropropionyl chloride in DMF in the presence of potassium carbonate yielded compounds **2** and **3** respectively. In the same manner using 4-florobenzoyl chloride, 4-methoxy benzoyl chloride, p-toluoyl chloride and 2-thiophenecarbonyl chloride in chloroform in the presence of triethyl amine yielded compounds **4-7** respectively. The structures of compounds **2-7** were established on the basis of the spectral data. For example, the ^{1}H NMR spectra of compound **3** showed characteristic triplet-quartet pattern for ethyl ester; the triplet appeared at 1.4 ppm while the quartet appeared at 4.4 ppm. In addition to two new triplets of (-**CH$_2$CH$_2$**Cl) at 3.0 and 3.9 ppm. The neuclophilic substitution of alkyl halides

with amines was reported in several publications[25-28], in the present study compounds **8-13** were obtained through refluxing mixture of chloroacetamido derivative **2** or chloro propanamido derivative **3** with phenyl piperazine, benzyl piperazine and morpholine in DMF in the presence of triethyl amine. The structures of these compounds were based on the basis of their spectral data. For example, in the ^{1}H NMR spectra of compounds **8**, **9**, **11**and **12** the piperazine protons appeared at the expected regions (Experimental part). In addition to a singlet signal for(benzylic- **$\underline{CH_2}$**) at 3.4-3.6 ppm in case of compounds **9** and **12**. Regarding compounds **10** and **13**, the ^{1}H NMR spectra showed characteristic two triplets signals of morpholine ring at 2.6 and 3.7-3.8 ppm.



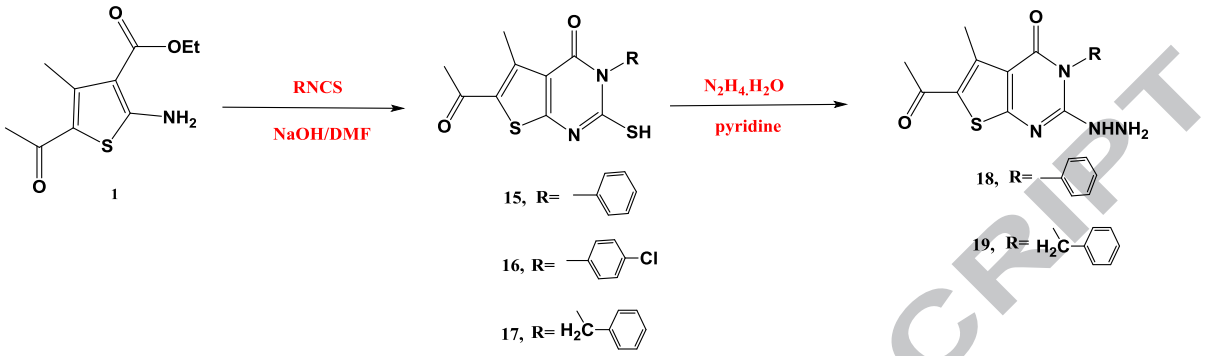
scheme 1: the synthesis of compounds 2-14

In this study, it was unexpected surprise to obtain compound **14** instead of **14a** or **14b** when compound **2** or compound **3** was treated with hydrazine hydrate. The reaction were thought to proceed on either pathway (a) where the neuclophilic attack occurs on the carbonyl carbon of ester group and results in the formation of the corresponding acid hydrazide **14a** or pathway (b) where the attack takes place on chloromethyl group associated with elimination of HCl to produce **14b**. Although, the neuclophilic substitution reaction on C-2 of amino thiophene is considered a difficult reaction due to the electron donation effect of the amino group, once the ring activated by electron withdrawing substitution at C-3 such as carboxamido[29] or ester groups it becomes activated towards neuclophilic attack on C-2 .In a similar way, it was reported that the aminoester thiophene underwent substitution reaction with aniline[30] and this could be the reasonable explanation in our case. The structure of compound **14** was assigned according to the spectral analysis, as the ^{1}H NMR showed the triplet-quartet pattern of the ethyl group in addition to two D_2O exchangeable singlet signals at 5.2 and 6.2 ppm for the NH and NH_2 protons, on the other hand the singlet peak of -**CH$_2$**Cl and the two triplets of -**CH$_2$CH$_2$**Cl of compounds **2** and **3** respectively were disappeared from the spectra . Also, the two nitrogen atoms of hydrazino group appeared as two signals at 200 and 406 ppm in the ^{15}N NMR spectra.



On the other hand, treatment of compound **1** with phenylisothiocyanate, 4-chloro phenyl isothiocyanate and benzyl isothiocyanate in DMF in the presence of sodium hydroxide followed by acidification with diluted acetic acid afforded thienopyrimidines derivatives **15-17**. Compounds **15** and **17** reacted with hydrazine hydrate in pyridine to give the corresponding hydrazino derivatives **18** and **19**. The structure of the compounds was established on the basis of the spectral data. For example, the ^{1}H NMR of compounds **15-17** revealed the disappearance of the two signals of ester group, in addition to the

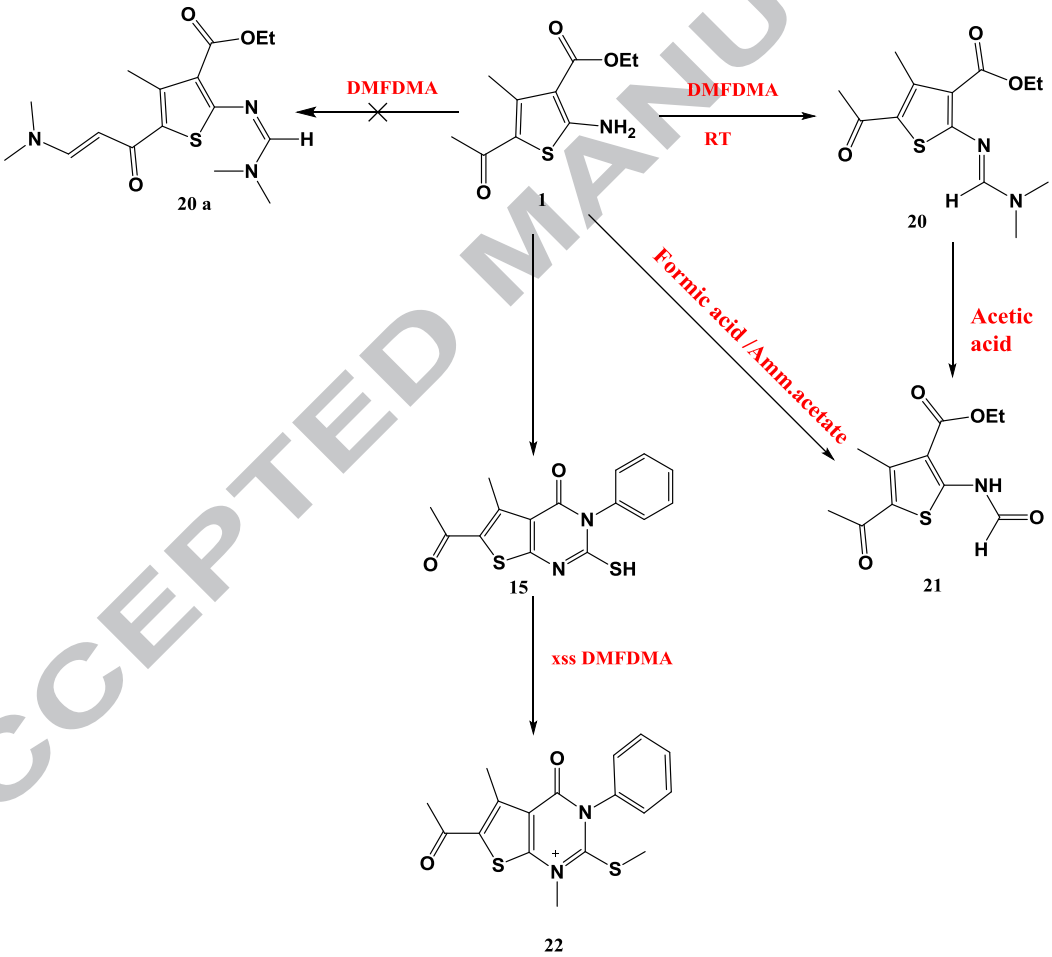
appearance of the aromatic protons of phenyl and benzyl moieties at their expected region (Experimental part).



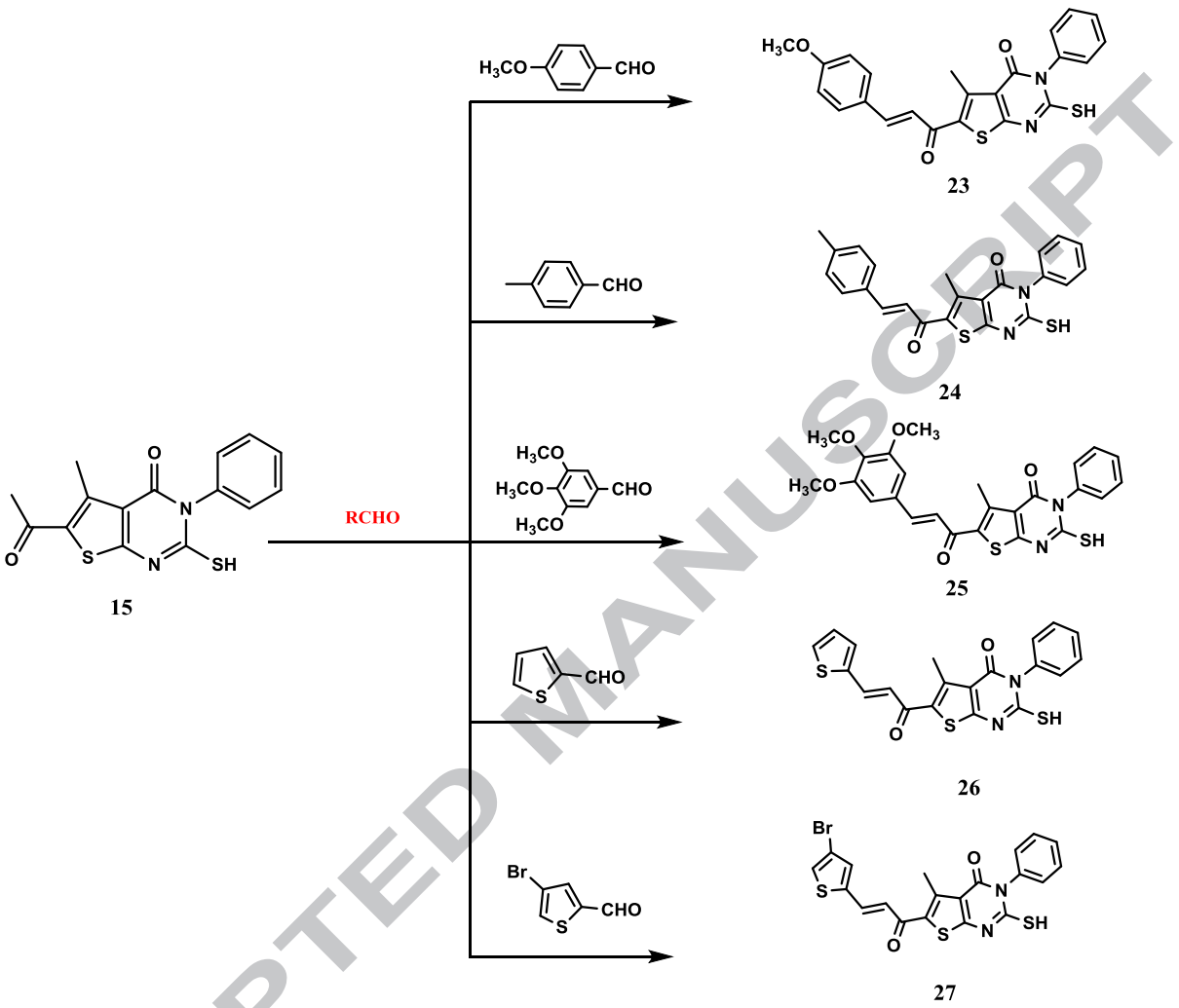
scheme 2: synthesis of compounds 15-17

The *N*,*N*-dimethyl formamide dimethyl acetal known as DMFDMA is a very useful reagent for the synthesis of various heterocyclic compounds. Several procedures have been reported for the condensation of DMFDMA with carbonyl compounds to yield the corresponding enaminone [31, 32]. It was also reported that DMFDMA can form formamidine derivatives by reacting with amines[33]. In this study, several conditions were tried including the reported procedure by Prasad *et al.*[34] to synthesize compound **20a** but only compound **20** was obtained when compound **1** was treated with DMFDMA. The formamidine derivative **20** underwent acidic hydrolysis by using glacial acetic acid to afford formmamide derivative **21** which was also obtained through treatment of compound **1** with formic acid in the presence of ammonium acetate. The product obtained from the two approaches was found to be identical with respect to melting point, thin layer chromatography (TLC) and spectral data. Another function of DMFDMA is methylation of sulfur and nitrogen [35], so when compound **15** refluxed with DMFDMA gave rise to S,N-dialkylated derivative **22**. The structure of these compounds was established on the basis of their spectral data. For example, the ^{1}H NMR spectra of compound **20** showed singlet peak at 3.3 ppm of (($\underline{\mathbf{CH_3}}$)$_2$ N-) and another singlet peak at 7.7 ppm of (-N=$\underline{\mathbf{CH}}$-) which both disappeared in the ^{1}H NMR spectra of compound **21**, instead it showed two new singlet peaks at 8.6 and 11.6 ppm for (-N$\underline{\mathbf{H}}$CHO) and (-NHC$\underline{\mathbf{H}}$O).

Several synthetic procedures have been reported for preparation of chalcones[36-38].In this study chalcones **23-27** were synthesized by the reaction between compound **15** and appropriate aldehyde in two reaction conditions ethanol/10% NaOH solution[39] and in sodium ethoxide solution. The structure of the compounds was established on the basis of their spectral data. The ^{1}HNMR showed two new doublet peaks at 7.1-7.4 and 7.5-7.9 ppm characteristic for the protons of alkene as well as the disappearance of the singlet peak of protons of acetyl group at 2.5ppm.



scheme 3: Synthesis of compounds 20-22



scheme 4: Synthesis of compounds 23-27

2.2.Target prediction

Target identification plays a central role in the drug design process. There are many online open access resources for target hunting in drug discovery such as BindingDB, DrugCentral and Pharmmapper. So, we tried to investigate the potential targets of our newly synthesized compounds using Pharmmapper, which is a freely accessed web-server designed to identify potential target candidates for the given probe small molecules (drugs, natural products, or other newly discovered compounds with binding targets unidentified) using pharmacophore mapping approach[40].The web server ranked the top 300 potential targets for each compound based on the binding affinity to the target compounds. Six targets, involved in cancer therapy are common between the tested compounds. The fit scores with the top six targets are listed in **Table 1**

Table 1:The fit scores of the tested compounds with top six targets demonstrated by pharmmapper

Compound NO.	DNA polymerase (1T8E)	Insulin like growth factor 1 receptor (1IGR)	Tyrosine Protein Kinase HCK (2C0O)	Alk tyrosine kinase receptor (2YS5)	Thymidylate synthase 1 (1BSP)	Thymidylate synthase (1TVV)
2	6.0	5.9	5.0	6.8	3.9	-*
3	5.9	5.8	-*	6.8	4.1	-*
4	5.9	6.4	5.4	6.8	4.1	5.9
5	5.9	5.9	-*	6.2	-*	5.1
6	5.9	6.5	5.9	6.3	4.0	5.6
7	5.8	5.1	-*	-*	-*	5.3
8	5.7	5.2	6.6	6.7	4	5.9
9	5.8	5.8	6.8	6.2	-*	5.9
10	5.8	5.9	-*	6	-*	3.4
11	6.1	5.8	6.7	6.5	-*	5.9
12	5.9	5.8	6.4	6.4	3.8	5.8
13	5.9	5.8	-*	6.0	-*	4.5
15	5.2	5.6	-*	5.3	2.9	-*
16	5.2	5.6	5.9	5.9	3.7	4.3
17	5.7	5.6	-*	6.3	2.9	-*
20	5.3	5.9	-*	5.5	-*	-*
21	5.1	5.9	-*	5.8	-*	-*
22	5.9	5.7	-*	6.9	3.7	-*
23	5.9	5.7	5.2	5.9	-*	5.8
24	5.9	5.6	6.0	6.6	3.9	6.6
25	6.0	6.0	5.8	6.2	3.9	6.1
26	5.9	-*	-*	6.0	3.9	5.8
27	5.9	5.9	6.2	7.0	4.3	6.6

*-This target is not included in top 300 target of this compound.

2.3.Biological activity

2.3.1. *in vitro* anti proliferative activity

Twenty three compounds **1-13**, **15-17** and **23-27** were selected to be screened for their anticancer activity *in vitro* against five representative cell lines of hepatocellular carcinoma (liver) HepG-2, epdermoid carcinoma (larynx) Hep-2, mammary gland (breast) MCF-7, human prostate cancer PC-3 and epitheloid cervix carcinoma HeLa using the standard MTT method[41] The cell lines were obtained from ATCC *via* Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. Doxorubicin was used as standard anticancer drug for comparison. The results of cytotoxic activity is reported in **Table 2.**

MTT assay is a standard colorimetric assay for measuring cell growth. It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. In brief, yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenases of living cells. A suitable solvent is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength. When the amount of purple formazan produced by cells treated with an agent is compared with that produced by unreacted control cells, the effectiveness of the agent in causing death of cells can deduced, through the production of a dose response curve[42].

The obtained results showed variable degree of inhibitory activity towards the five tested human tumor cell lines. Six compounds **15**, **16**, **17**, **22**, **24** and **25** among the tested compounds showed good inhibitory activity towards all tested cell lines. (The dose response curves of the six active compounds and doxorubicin are reported in **Fig.1**) As for activity against hepatocellular carcinoma HepG-2 and mammary gland (breast) MCF-7 cell line the highest activity was displayed by compounds **22** and **24** which showed the percentage viability IC_{50} at 10.07 and 7.99 µM respectively for hepatocellular carcinoma cell line HepG-2 while in case of mammary gland (breast) MCF-7 cell line IC_{50} appeared at 7.17 and 6.84 µM respectively .

Further interpretation of the results revealed that in the epdermoid carcinoma (larynx) Hep-2 , human prostate cancer PC-3 and epitheloid cervix carcinoma HeLa cell lines compound **24** showed the highest activity with IC_{50} at 5.40 µM, 7.71 µM and 4.30 µM respectively, being more potent than doxorubicin which showed IC_{50} at 6.32 µM, 8.87 µM and 5.57 µM respectively. Compounds **17** and **22** also showed remarkable inhibitory activity against the three cell lines.

Table 2: Cytotoxicity (IC_{50}) of tested compounds on different cell lines**.**

compound	In vitro cytotoxicity IC_{50} (µM)*				
	HepG-2	Hep-2	MCF-7	PC-3	HeLa
DOX	4.50±0.2	6.32±0.3	4.17±0.2	8.87±0.6	5.57±0.4
2	91.63±5.0	89.69±4.9	82.14±4.7	>100	87.60±4.8
3	52.89±3.7	42.35±2.8	53.42±3.3	41.48±2.7	27.47±2.0
4	74.24±4.3	86.52±4.5	68.90±4.1	>100	72.92±4.3
5	>100	97.60±5.3	94.17±5.0	>100	>100
6	38.47±2.7	51.14±3.2	38.19±2.6	42.92±2.8	31.13±2.4
7	78.35±4.5	81.23±4.4	72.18±4.2	87.36±4.8	67.08±3.9
8	51.18±3.4	71.14±3.7	47.71±3.0	64.71±4.0	46.67±3.1
9	83.00±4.7	88.96±4.8	77.54±4.5	>100	73.91±4.5
10	64.10±4.0	76.93±3.9	63.40±3.8	71.81±4.5	56.59±3.5
11	58.77±3.8	57.55±3.4	56.42±3.5	55.94±3.7	39.03±2.5
12	70.77±4.1	46.84±3.0	64.54±3.9	64.70±4.4	29.82±2.2
13	45.78±3.1	73.21±3.8	45.95±2.8	52.24±3.5	55.54±3.3
15	14.22±1.3	15.88±1.3	8.16±0.8	12.19±1.1	11.53±1.1
16	23.57±1.8	19.87±1.5	18.85±1.5	15.32±1.3	12.62±1.2
17	15.91±1.4	9.58±0.7	14.20±1.2	9.05±0.9	8.03±0.6
20	35.70±2.5	33.31±2.4	37.26±2.3	36.35±2.4	19.93±1.7
21	41.48±2.9	65.01±3.4	41.59±2.6	48.72±3.2	40.82±2.7
22	10.07±1.0	11.29±1.1	7.17±0.6	10.92±1.1	9.31±0.9
23	32.75±2.3	39.50±2.6	28.90±2.0	28.95±2.2	22.72±1.8
24	7.99±0.6	5.40±0.3	6.84±0.4	7.71±0.5	4.30±0.3
25	20.58±1.7	22.05±1.6	17.14±1.4	16.38±1.5	12.88±1.1
26	26.42±1.9	25.73±1.8	24.02±1.7	18.43±1.6	15.41±1.3
27	30.06±2.1	31.33±2.3	25.84±1.9	27.71±1.9	17.75±1.5

*** IC_{50} (µM)** : 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic) , **DOX** : Doxorubicin

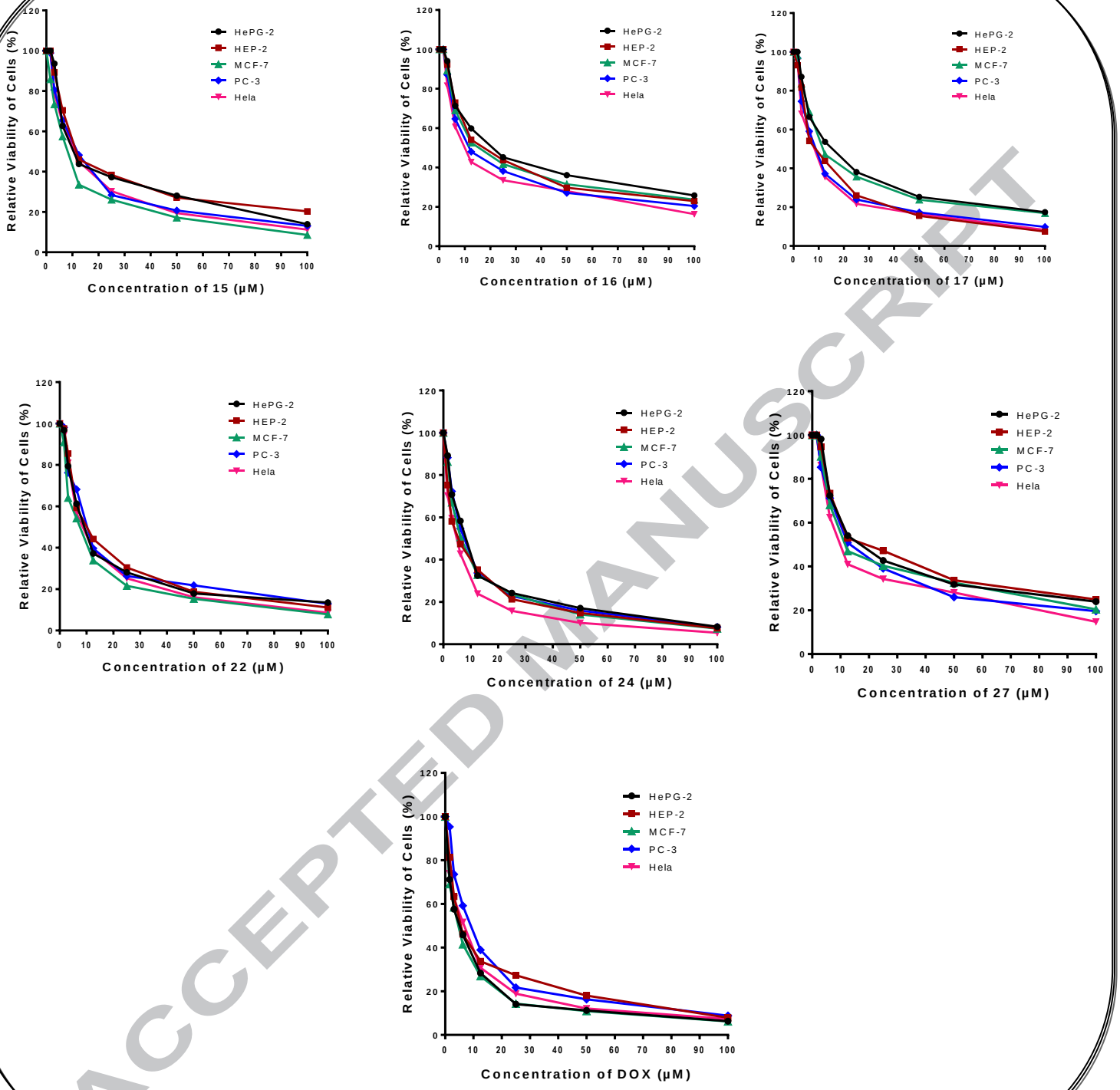


Fig.1 : The dose response curves of the six active compounds and doxirubicin.

2.3.2. DNA/ methyl green assay

The mechanism of many antitumor agents involves binding with DNA. A variety of methods have been utilized for the interaction of small molecular weight compounds with DNA as DNA binding assay and methyl green binding assay. So, compounds **15**, **16**, **17**, **24** and **25** were selected to test their DNA binding affinity compared to doxorubicin as positive control.

DNA/methyl green assay is a colorimetric microassay method for the detection of DNA binding agents. The methyl green dye reversibly binds DNA to form colored DNA/methyl green complex. This color is stable at neutral pH. Upon displacement of methyl green from DNA by DNA binding compounds, facilitates the addition of H_2O molecule to the dye, which results in formation of the colorless carbinol, followed by spectrophotometrically a decrease in absorbance at 630 nm[43]. Results from DNA binding assay (**Table 3**) revealed that compounds **15**, **16** and **17** showed the highest affinity for DNA, which was demonstrated by measuring IC_{50} (concentration required for 50% decrease in the initial absorbance of the DNA/methyl green solution). While compounds **24** and **25** showed moderate and weak DNA binding affinity respectively.

Table 3: DNA/methyl green colorimetric assay of the tested compounds

Compounds. NO.	DNA/methyl green (IC_{50} µM)*
15	**36.06±2.1**
16	**33.98±1.8**
17	**27.31±1.4**
24	**42.72±2.5**
25	**51.40±3.2**
***DOX**	**32.35±1.6**

IC_{50}values represent the concentration (mean ± SD, n = 3–5 separate determinations) required for a 50% decrease in the initial absorbance of the DNA/methyl-green solution.

*DOX: Doxorubicin

2.3.3. Enzyme assay

In order to explain the observed anti proliferative activity and based on the pharmmapper results, three enzymes; DNA polymerase, thymidylate synthase and tyrosine kinase were selected to screen the enzyme inhibition activity of the synthesized compounds. These enzymes are known to be targets of anticancer chemotherapeutic agents[44-48].

Five compounds with the highest antitumor activities(**15**,**16**,**17**,**24** and **25)** were selected to be evaluated against human DNA polymerase beta assay kit, human thymidylate synthase ELISA kit and protein tyrosine kinase kit. The enzyme inhibition percentages and IC_{50} values were calculated at serial dilutions of the tested compounds.(**Table 4 and 5**).

The tested compounds showed variable enzyme inhibition percentages for the three enzymes. The results showed that the highest inhibition percentages were for DNA polymerase ranging between 80 to 92% followed by thymidylate synthase with inhibition percentages ranging between 75-92%. On other hand, tyrosine kinase showed the lowest inhibition percentages ranging between 71-85%.

Compounds **16** (IC_{50}-1.42µM) and **17** (IC_{50}-0.9µM) showed the highest inhibitory activity against DNA polymerase compared to other tested compounds, while compounds **17** (IC_{50}-27.97 µM) and **25 (**IC_{50}-29.19 µM) showed the highest inhibitory activity against thymidylate synthase. Meanwhile, compounds **17** (IC_{50}-73.4µM) and **24** (IC_{50}-135.5µM) were the most potent inhibitors for tyrosine kinase.

Table 4: Enzyme inhibition percentages of serial dilutions of the tested compounds against DNA polymerase, thymidylate synthase and tyrosine kinase.

Compound NO.	Log conc.	Enzyme inhibition percentage		
		DNA polymerase	Thymidylate synthase	Tyrosine kinase
15	3	80.28273	68.18881	NT
	2	65.94021	35.02651	71.77871
	1	56.25092	18.76176	25.56022
	0	12.57547	0.803831	9.803922
	-1	NT	NT	1.68067
16	3	92.32219	84.37489	NT
	2	82.16757	53.06995	73.65546
	1	69.63628	28.40773	28.5014
	0	42.74775	1.060373	13.23529
	-1	NT	NT	0.07003
17	3	90.32838	90.36942	NT
	2	68.75276	74.10638	85.07003
	1	61.87601	38.41286	53.0042
	0	52.54013	3.43766	17.507
	-1	NT	NT	3.361345
24	3	85.57503	75.88507	NT
	2	74.61346	36.46314	79.90196
	1	63.95229	16.82914	37.87115
	0	27.35974	0.410467	15.68627
	-1	NT	NT	1.330532
25	3	83.91989	92.53805	NT
	2	65.94021	67.16265	77.03081
	1	57.42895	42.55174	30.60924
	0	34.63407	1.864204	11.69468
	-1	NT	NT	1.2605

NT : this compound is not tested at this concentration.

Table 5 :IC_{50} values of the tested compounds against DNA polymerase, thymidylate synthase and tyrosine kinase.

Compound NO.	Enzymatic IC_{50} (µM)		
	DNA polymerase	Thymidylate synthase	Tyrosine kinase
15	21.04	242.04	316.8
16	1.42	63.26	248.5
17	0.9	27.96	73.4
24	6.38	164.2	135.5
25	6.75	29.19	202.7

2.2.4.Structure activity relationship (SAR)

The replacement of chloroacetamido group in compound **2** with chloropropanamido group in compound **3** enhanced the antitumor activity against all tested cell lines and therefore the activity of compounds **11, 12** and **13** is much better than compounds **8**, **9** and **10** .The para substitution on the phenyl moiety with floro or methyl group enhanced the antitumor activity as seen in compounds **4** and **6** respectively, meanwhile, the para substitution of phenyl with methoxy group greatly reduced the antitumor activity as seen in compound **5**. Also, the isosteric substitution of phenyl ring of compound **4** with thiophene ring in compound **7** greatly reduced the activity.

The cyclization of *o*-amino ester thiophene into thienopyrimidines greatly enhanced the activity as seen in compounds **15**-**27**.The substitution of phenyl ring at N-3 of thienopyrimidine ring with benzyl moiety in compound **17** enhanced the antitumor activity. The S,N-dimethylation of compound **15** highly enhanced the antitumor activity as seen in compound **22**.

Regarding chalcones (**23-27**), replacement of para methoxy phenyl group in compound **23** with *p*-tolyl group in compound **24** or with 3,4,5-trimethoxy phenyl moiety in compound **25** greatly enhanced the antitumor activity. On the other hand, the isosteric replacement of phenyl moiety in compounds **(23-25)** with thiophene moiety in compound **26** or with 4-bromo thiophene moiety in compound **27** reduced the activty against the tested cell lines.

3.Conclusion

New thiophene and thienopyrimidine derivatives have been synthesized and their antitumor activity and enzyme inhibitory activity against DNA polymerase, thymidylate synthase and tyrosine kinase have been evaluated. Among the tested compounds, **15**,**16**,**17**,**24** and **25** were identified as the most active antitumor agents against the five cell lines tested comparable to doxorubicin. Moreover, compound **17** was identified as the most potent inhibitor for the three tested enzymes with IC_{50} 0.9, 27.9 and 73.4 µM respectively. Also, compounds **16**,**24** and **25** showed high enzyme inhibition percentages

against the screened enzymes .So, this study revealed that compounds **16,17,24 and 25** could be promising leads for further development of more potent antitumor agents.

4.Experimental

4.1.General

Ethyl 5-acetyl-2-amino-4-methylthiophene-3-carboxylate **1** was prepared following the procedure reported by Abu-Hashem[24]. All the reagents and solvents were obtained from commercial suppliers and used without any purification. The reactions were monitored by using Fluka silica gel TLC alumminium cards (0.2 mm). Compounds were visualized by using 254 nm UV lamp. Melting points (°C) were recorded using a Stuart digital melting point apparatus SMP30 and are uncorrected. The IR spectra (KBr) were recorded on Mattson 5000 FT IR spectrophotometer in (ν cm^{-1}) in Central Laboratory Unit, Faculty of Pharmacy, Mansoura University. ^{1}H NMR , ^{13}C NMR and ^{15}N NMR were recorded on Burker 400 MHz FT NMR spectrometer by using (DMSO-d_6) or ($CDCl_3$) as solvents with chemichal shift being expressed in δ (ppm) with tetramethylsilane (TMS) as standard. The mass spectrum was carried out on direct probe controller inlet part to single quadropole mass analyzer in (thermo scientific GCMS) model (ISQ LT) using thermo x-calibur software at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo. The enzyme inhibition assay was performed in confirmatory diagnostic unit, VACSERA-EGYPT.

4.2. General procedure for the synthesis of compound (**2** and **3**)

A mixture of aminothiophene **1** (2.27gm, 10 mmol) and K_2CO_3(1.38 gm, 10 mmol) in DMF (20 mL) was stirred in ice bath for 10 minutes. Then (30 mmol) chloroacetyl chloride or chloropropionyl chloride was added dropwise to the mixture, which is left stirring at room temperature overnight. The mixture was then poured into crushed ice. The formed yellowish white precipitate was separated by filtration, washed several times with water and crystallized from aqueous ethanol.

4.2.1. Ethyl 5-acetyl-2-(2-chloroacetamido)-4-methylthiophene-3-carboxylate(**2)**

Yield:80% ; mp: 170 ˚C ; **^{1}H NMR** (δ, ppm, $CDCl_3$): 1.4(t, 3H, J=13.6, $\underline{CH_3}CH_2COO$-), 2.5 (s, 3H, $\underline{CH_3}CO$-), 2.7(s, 3H, CH_3-thiophene), 4.1(s, 2H, -Cl-$\underline{CH_2}$-CONH-), 4.4 (q, 2H, $CH_3\underline{CH_2}COO$-), 12.4 (s, 1H, D_2O exchangeable, $\underline{NH}$-CO-). **^{13}C NMR** (δ, ppm, $CDCl_3$) :14.2 (CH_3 ester), 16 (CH_3 thiophene), 30 (CH_3 acetyl), 42.2 (-$\underline{CH_2}$-CONH-), 61.6 ($CH_3\underline{CH_2}COO$-), 116.1, 127.6, 143.6, 150.3 (thiophene quaternary carbons), 164.5 ($\underline{CO}O$-), 166 (-$\underline{CO}$NH-), 191.5 ($\underline{CO}CH_3$). **MS** m/z (%) Calcd for $C_{12}H_{14}ClNO_4S$; 303.76, found; 303 (2.9,M^+).

4.2.2. Ethyl 5-acetyl-2-(3-chloropropanamido)-4-methylthiophene-3-carboxylate **(3)**

Yield:70% ; mp:108 ˚C ; IR (KBr, ν, cm^{-1}) : 3252 (NH amide), 1716 (C=O acetyl), 1667 (C=O amide).**^{1}H NMR** (δ, ppm, $CDCl_3$): 1.4(t, 3H, J=13.6, $\underline{CH_3}CH_2COO$-), 2.5 (s, 3H, $\underline{CH_3}CO$-), 2.7(s, 3H, CH_3-thiophene), 3.0(t, 2H, J=11.6, -CH_2-$\underline{CH_2}$-CONH-), 3.9 (t, 2H, J=11.6, Cl-$\underline{CH_2}$-CH_2-CONH-), 4.4 (q, 2H, $CH_3\underline{CH_2}COO$-), 11.7 (s, 1H, D_2O exchangeable, $\underline{NH}$-CO-). **^{13}C NMR** (δ, ppm, $CDCl_3$) :14.2 (CH_3 ester), 16 (CH_3 thiophene),30 (CH_3 acetyl),38 (-$\underline{CH_2}$-CONH-),39 (Cl-$\underline{CH_2}$-),61 ($CH_3\underline{CH_2}COO$-),115,127,143,151 (thiophene quaternary carbons), 166 ($\underline{CO}O$-), 167 (-$\underline{CO}$NH-), 191 ($\underline{CO}CH_3$). **MS** m/z (%) Calcd for $C_{13}H_{16}ClNO_4S$; 317.78, found; 319 (66.05,M+2), 317 (100,M^+).

4.3.General procedure for the synthesis of Ethyl 5-acetyl-4-methyl-2-(4-substituted benzamido)thiophene-3-carboxylate (**4-6**)

A mixture of aminothiophene **1** (2.27gm,10 mmol), triethyl amine (0.3 mL, 3mmol) and the appropriate 4-substituted benzoyl chloride (40 mmol) in chloroform (15 mL) was stirred at room temperature for 24 h. Then the reaction mixture was distilled under reduced pressure and the residue obtained was washed several times with diluted aqueous ammonia solution then crystallized from methanol.

4.3.1. Ethyl 5-acetyl-2-(4-fluorobenzamido)-4-methylthiophene-3-carboxylate(**4**).

Yield:70% ; mp:168 ˚C ; **^{1}H NMR** (δ, ppm, $CDCl_3$) :1.4 (t, 3H, J=14.4, $\underline{CH_3}CH_2COO$) ,2.5 (s,3H,$\underline{CH_3CO}$-) ,2.8 (s,3H,CH_3-thiophene), 4.4 (q, 2H, $CH_3\underline{CH_2}COO$-), 7.2 (ddd, 2H ,Ar-H), 8.0 (ddd, 2H, Ar-H), 12.6 (s, 1H, D_2O exchangable-CONH-) .**^{13}C NMR** (δ, ppm,

$CDCl_3$): 14.2 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 61 ($CH_3\underline{CH_2}COO$-), 115,127,143.8,152 (thiophene quaternary carbons),116.2,116.4 ,128, 130.1 130.2 ,163 (phenyl aromatic carbons), 164 (-<u>CO</u>NH-), 167 (-<u>CO</u>O-), 191 ($CH_3\underline{CO}$-). **MS** m/z(%): Calcd for $C_{17}H_{16}FNO_4S$; 349.38, found; 349 (100, M^+).

4.3.2. Ethyl 5-acetyl-2-(4-methoxybenzamido)-4-methylthiophene-3-carboxylate (**5**).

Yield:45% ; mp:205˚C ; **^{1}H NMR** (δ, ppm, $CDCl_3$): 1.4 (t, 3H, J=14.4, $\underline{CH_3}CH_2COO$), 2.5 (s, 3H, $\underline{CH_3}CO$-), 2.8 (s, 3H, CH_3-thiophene), 3.9 (s, 3H, -$\underline{OCH_3}$), 4.4 (q, 2H, $CH_3\underline{CH_2}COO$-), 7.0 (d, 2H, J=8.8, Ar-H), 8.0 (d,2H, J=8.4, Ar-H), 12.5 (s,1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$): 14.2 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 55 (-O<u>C</u>H_3) ,61($CH_3\underline{CH_2}COO$-) , 114.8 ,127, 143 ,152.7 (Thiophene quaternary carbons) , 114.3, 123.9, 129.6, 163.5 (phenyl aromatic carbons) ,163 (-<u>CO</u>NH-), 167 (-<u>CO</u>O-), 191 ($CH_3\underline{CO}$-). **MS** m/z (%): Calcd for $C_{18}H_{19}NO_5S$; 361.41, found; 361(100, M^+).

4.3.3. Ethyl 5-acetyl-4-methyl-2-(4-methylbenzamido)thiophene-3-carboxylate (**6**).

Yield:65% ; mp:159˚C ; **^{1}H NMR** (δ, ppm, $CDCl_3$): 1.4 (t, 3H, J=14.4, $\underline{CH_3}CH_2COO$) ,2.47 (s, 3H, CH_3CO-), 2.5 (s, 3H, CH_3-thiophene), 2.8 (s, 3H, -CH_3-phenyl) ,4.4 (q, 2H, $CH_3\underline{CH_2}COO$-), 7.3(d, 2H, J=8, Ar-H), 7.9 (d, 2H , J=8.4, Ar-H), 12.6 (s, 1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14.2 (CH_3 ester), 16.15 (CH_3 thiophene), 21.7 (CH_3-phenyl), 30 (CH_3 acetyl), 61($CH_3\underline{CH_2}COO$-), 115 ,126.8, 143 ,152 (thiophene quaternary carbons), 127.6 ,128.8 ,129.1 ,129.8 (phenyl aromatic carbons),164.2 (-<u>CO</u>NH-) ,167.13 (-<u>CO</u>O-) ,191.17 ($CH_3\underline{CO}$-). **MS** m/z (%):Calcd for $C_{18}H_{19}NO_4S$; 345.41, found; 345(100, M^+).

4.4. Synthesis of ethyl 5-acetyl-4-methyl-2-(thiophene-2-carboxamido)thiophene-3-carboxylate (**7**)

A mixture of aminothiophene **1** (2.27gm, 10 mmol), triethyl amine (0.3 mL, 3mmol) and 2-thiophene carbonyl chloride (2.9gm, 20 mmol) in chloroform (15 mL) was stirred at room temperature for 24 h. Then the reaction mixture was distilled under reduced pressure and the residue obtained was washed several times with diluted aqueous ammonia solution then crystallized from methanol.

Yield: 80% ; mp:158°C ; **^{1}H NMR** (δ, ppm, $CDCl_3$): 1.4 (t, 3H, J=14.4, CH_3CH_2COO), 2.5 (s, 3H, CH_3CO-), 2.8 (s, 3H, CH_3-thiophene), 4.4 (q, 2H, CH_3CH_2COO-), 7.2 (t, 1H ,J=8.8 ,Ar-H), 7.7 (d, 1H, J=4.8, Ar-H), 7.8 (d, 1H, J=3.6, Ar-H) 12.5 (s, 1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$): 14.2 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 61(CH_3CH_2COO-) , 115 ,127, 143.9,152 (thiophene quaternary carbons), 128,130,132,136.7 (thiophene carbons), 159 (-CONH-), 167 (-COO-), 191.6 (CH_3CO-). **MS** m/z (%):Calcd for $C_{15}H_{15}NO_4S_2$;337.41, found; 337(21.2, M^+).

4.5.General procedure for synthesis of compounds (**8-13**)

A mixture of compound **2** or compound **3** (10 mmol), triethyl amine (0.3mL, 3 mmol) and appropriate 2ry aliphatic amine (10 mmol) in DMF (10 mL) was refluxed for 8 h. The reaction mixture was cooled and poured into crushed ice and the formed precipitate was filtered, washed with water and crystallized from ethanol.

4.5.1. Ethyl 5-acetyl-4-methyl-2-(2-(4-phenylpiperazin-1-yl)acetamido)thiophene-3-carboxylate (**8**).

Yield:50% ; mp:189°C ; **IR** (KBr, ν, cm^{-1}) : 3440-3447 (NH amide), 1709-1716 (C=O acetyl), 1740 (C=O ester), 1660-1669 (C=Oamide).**^{1}H NMR** (δ, ppm, $CDCl_3$): 1.3 (t ,3H , J=13.6, CH_3CH_2COO), 2.4 (s, 3H, CH_3CO-), 2.7(s, 3H, CH_3-thiophene), 2.76 (br s, 4H, piperazine), 3.2 (br s, 4H, piperazine), 3.3 (s, 2H, -NHCOCH_2-), 4.3 (q, 2H, CH_3CH_2COO-), 6.8 (t, 1H, J=13.6, Ar-p-H), 6.9 (d, 2H, J=7.6, Ar-H), 7.2 (m, 2H, Ar-H) 12.6 (s, 1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14.2 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 49, 53 (carbons of piperazine ring), 61 (-NHCOCH_2-) 61.2 (CH_3CH_2COO-), 116, 127, 143 ,150 (thiophene quaternary carbons), 115,8, 129.32 (aromatic phenyl carbons),165.22 (-CONH-), 169 (-COO-), 191.7 (CH_3CO-). **MS** m/z (%): Calcd for $C_{22}H_{27}N_3O_4S$; 329.53, found; 429(100, M^+).

4.5.2. Ethyl 5-acetyl-2-(2-(4-benzylpiperazin-1-yl)acetamido)-4-methylthiophene-3-carboxylate (**9**).

Yield:77% ; mp:183°C ; **IR** (KBr, ν,cm^{-1}) : 3440-3447 (NH amide), 1709-1716 (C=O acetyl), 1740 (C=O ester), 1660-1669 (C=O amide).^{1}H NMR (δ, ppm, $CDCl_3$)**:** 1.4 (t, 3H, J=14, CH_3CH_2COO), 2.5 (s, 3H, CH_3CO-), 2.7(s, 3H, CH_3-thiophene), 2.6 (m, 8H,

piperazine), 3.3 (s, 2H, -NHCO$\underline{CH_2}$-), 3.6 (s, 2H, -$\underline{CH_2}$-aromatic), 4.4 (q, 2H, $CH_3\underline{CH_2}$COO-), 6.8 (t, 1H, J=13.6, Ar-p-H), 6.9 (d, 2H , J=7.6, Ar-H), 7.3-7.4(m, 5H, Ar-H) 12.5 (s, 1H, D_2O exchangeable -CO$\underline{NH}$-). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14.3 ($\underline{CH_3}$ ester), 16.15 ($\underline{CH_3}$ thiophene), 30 ($\underline{CH_3}$ acetyl), 52 , 53 (carbons of piperazine ring), 61.1 (-NHCO$\underline{CH_2}$-), 61.1($CH_3\underline{CH_2}$COO-), 62.8 (-$\underline{CH_2}$-aromatic), 115 ,127, 143 ,150 (thiophene quaternary carbons), 126,128,129.2,137.7 (aromatic phenyl carbons),165 (-$\underline{CO}$NH-) ,169.5 (-$\underline{CO}$O-), 191.7 ($CH_3\underline{CO}$-). **MS** m/z (%): Calcd for $C_{23}H_{29}N_3O_3S$; 443.56, found; 443(100, M^+).

4.5.3. Ethyl 5-acetyl-4-methyl-2-(2-morpholinoacetamido)thiophene-3-carboxylate (**10**).

Yield:66% ; mp:190°C; ^{1}H NMR (δ, ppm, $CDCl_3$): 1.4 (t , 3H , J=14,$\underline{CH_3}CH_2$COO), 2.5 (s, 3H, $\underline{CH_3}$CO-), 2.7(s, 3H, $\underline{CH_3}$-thiophene), 2.6 (t, 4H, J=9.2, $(CH_2)_2$N-morpholine), 3.3 (s, 2H, -NHCO$\underline{CH_2}$-), 3.8 (t, 4H, J=9.2, $(CH_2)_2$O-morpholine), 4.4 (q, 2H, $CH_3\underline{CH_2}$COO-), 12.5 (s, 1H, D_2O exchangeable -CO$\underline{NH}$-). **^{13}C NMR** (δ, ppm, $CDCl_3$): 14.3 ($\underline{CH_3}$ ester), 16.15 ($\underline{CH_3}$ thiophene), 30 ($\underline{CH_3}$ acetyl), 53, 66(carbons of morpholine ring), 61.1 (-NHCO$\underline{CH_2}$-) 61.3($CH_3\underline{CH_2}$COO-) , 115 ,127, 143 ,150 (thiophene quaternary carbons) ,169 (-$\underline{CO}$NH-),171 (-$\underline{CO}$O-), 191 ($CH_3\underline{CO}$-). **MS** m/z (%): Calcd for $C_{16}H_{22}N_2O_5S$; 354.42, found; 454(100, M^+).

4.5.4. Ethyl 5-acetyl-4-methyl-2-(3-(4-phenylpiperazin-1-yl)propanamido)thiophene-3-carboxylate (**11**).

Yield:40% ; mp:108°C; **IR** (KBr, ν ,cm^{-1}) : 3440-3447 (NH amide), 1709-1716 (C=O acetyl), 1740 (C=O ester), 1660-1669 (C=O amide).**^{1}H NMR** (δ, ppm, $CDCl_3$): 1.3 (t , 3H, J=14, $\underline{CH_3}CH_2$COO), 2.5 (s, 3H, CH_3CO-), 2.72 (m, 4H, piperazine) 2.76(s, 3H, CH_3-thiophene), 2.8 (t, 4H, piperazine), 3.3 (t, 4H , J=9.6, -NHCO-$\underline{CH_2}$-$\underline{CH_2}$-), 4.3 (q , 2H, $CH_3\underline{CH_2}$COO-), 6.8 (t, 1H ,J=14.4, Ar-p-H), 6.9 (d, 2H, J=8.4, Ar-H), 7.2 (t, 2H, J=15.2, Ar-H), 12.3 (s, 1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 33 (- $\underline{CH_2}$-CONH-), 45 ($\underline{CH_2}$-CH_2-CONH-), 48, 53 (carbons of piperazine ring), 61 (-NHCO$\underline{CH_2}$-), 115 ,127, 143 ,151.3(thiophene quaternary carbons), 116, 119.72, 129, 151.73 (aromatic phenyl carbons

), 165.8 (-CONH-), 170 (-COO-), 191.8 (CH_3CO-). **MS** m/z (%): Calcd for $C_{23}H_{29}N_3O_4S$; 443.56, found; 443(100, M^+).

4.5.5. Ethyl 5-acetyl-2-(3-(4-benzylpiperazin-1-yl)propanamido)-4-methylthiophene-3-carboxylate (**12**).

Yield:64% ; mp:112°C; **IR** (KBr, ν, cm^{-1}) : 3440-3447 (NH amide), 1709-1716 (C=O acetyl), 1740 (C=O ester) ,1660-1669 (C=O amide) .**^{1}H NMR** (δ, ppm, $CDCl_3$): 1.3 (t, 3H, J=14, CH_3CH_2COO), 2.45 (s, 3H, CH_3CO-), 2.5 (br s, 8 H, piperazine), 2.6 (t, 2H, CH_2-CH_2-CONH-), 2.7(s, 3H, CH_3-thiophene), 2.9 (t, 2H, (- CH_2-CONH-), 3.4 (s, 2H,-CH_2-aromatic), 4.3 (q, 2H, CH_3CH_2COO-), 7.2-7.3 (m, 5H, Ar-H), 12.2 (s, 1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 33 (-CONH- CH_2-CH_2), 52.4 (-CONH-CH_2-CH_2-), 53,55, (carbons of piperazine ring) ,61(CH_3CH_2COO-),63.1 (-CH_2-aromatic), 115,138, 143 ,151(thiophene quaternary carbons), 126.7,127.1,128.2,129 (aromatic phenyl carbons), 165 (-CONH-), 170.4 (-COO-), 191.8 (CH_3CO-). **MS** m/z (%): Calcd for $C_{24}H_{31}N_3O_4S$; 457.59, found; 457(100, M^+).

4.5.6. Ethyl 5-acetyl-4-methyl-2-(3-morpholinopropanamido)thiophene-3-carboxylate (**13**).

Yield:73% ; mp:122°C; **IR** (KBr, ν, cm^{-1}) : 3440-3447 (NH amide), 1709-1716 (C=O acetyl), 1740 (C=O ester), 1660-1669 (C=O amide).**^{1}H NMR** (δ, ppm, $CDCl_3$): 1.3 (t ,3H , J=14, CH_3CH_2COO), 2.45 (s, 3H, CH_3CO-), 2.5-2.68(m, 8H, -CH2-CH2- + $(CH_2)_2$N-morpholine), 2.7 (s, 3H, CH_3-thiophene), 3.7 (t, 4H, J=9.2, $(CH_2)_2$O-morpholine) ,4.4 (q, 2H, CH_3CH_2COO-), 12.2 (s, 1H, D_2O exchangeable -CONH-). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14.3 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 33 (-CONH-CH_2-CH_2), 53 (-CONH-CH_2-CH_2-), 53.51 ,66.5 (carbons of morpholine ring), 61.1 (-NHCOCH_2-), 61.3(CH_3CH_2COO-), 115,126, 143,151 (thiophene quaternary carbons) ,165 (-CONH-), 170 (-COO-), 191.8 (CH_3CO-). **MS** m/z (%):Calcd for $C_{17}H_{24}N_2O_5S$; 368.45, found; 368(100, M^+).

4.6.Synthesis of ethyl 5-acetyl-2-hydrazinyl-4-methylthiophene-3-carboxylate (**14**).

A mixture of compound **2** or compound **3** (10 mmol) and hydrazine hydrate (1.5gm, 30 mmol) in ethanol (10 mL) was refluxed for 4 h. The reaction mixture was cooled, poured into crushed ice and left overnight. The obtained crystals were filtered, washed with water and crystallized from methanol.

Yield:30% ; mp:237°C; **IR** (KBr, ν, cm^{-1}) : 3415-3303 cm^{-1} ($NHNH_2$), 1700 cm^{-1} ($COCH_3$), 1744 cm^{-1} (-COO-). **^{1}H NMR** (δ, ppm, $CDCl_3$)**:** 1.3 (t, 3H, J=14, $\underline{CH_3}CH_2COO$), 2.15(s, 3H, CH_3CO-), 2.4(s, 3H, CH_3-thiophene), 4.4 (q, 2H, $CH_3\underline{CH_2}COO$-), 5.2 (s, 1H, D_2O exchangeable, thiophene-$\underline{NH}NH_2$), 6.2 (s, 2H, D_2O exchangeable, thiophene-$NH\underline{NH_2}$). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14.4 (CH_3 ester), 15.6 (CH_3 thiophene), 16.8 (CH_3 acetyl), 59.8($CH_3\underline{CH_2}COO$-) , 107.6 ,120, 133.1,144.79(thiophene quaternary carbons) ,162.9(-$\underline{CO}$O-) , 166.1 ($CH_3\underline{CO}$-). **N^{15}NMR** : 2 nitrogen atoms at 200 and 406 ppm. **MS** m/z (%): calc for $C_{10}H_{14}N_2O_3S$; 242.29, found; 242.06 (3.94, M^+).

4.7.General procedure for synthesis of compounds **(15-17)**

An equimolar mixture of aminothiophene **1** (2.27 gm, 10mmol), NaOH (0.4gm, 10mmol) and the appropriate isothiocyanate derivative (10 mmol) in DMF (20 mL) was refluxed for 6 h. The reaction mixture was cooled, poured into crushed ice, neutralized with diluted acetic acid and the solid formed was filtered and crystallized from ethanol.

4.7.1. 6-Acetyl-2-mercapto-5-methyl-3-phenylthieno[2,3-d]pyrimidin-4(3H)-one (**15**).

Yield:80% ; mp:246°C; **IR** (KBr, ν, cm^{-1}) :3394 (NH amide), 1669 (C=O amide), 1236 (C=S). **^{1}H NMR** (δ, ppm, DMSO-d_6): 2.5(s, 3H, -CO$\underline{CH_3}$), 2.7 (s, 3H, $\underline{CH_3}$-thiophene), 7.2 (d, 2H, Ar-H), 7.4 (m, 1H, Ar-H), 7.48 (t, 2H, Ar-H). **^{13}C NMR** (δ, ppm, DMSO-d_6):15 (CH_3 thiophene), 30 (CH_3 acetyl), 118, 128, 139, 154(thiophene quaternary carbons), 129, 131.04, 142, 150 (phenyl), 162 (C-SH), 176 (-$\underline{CO}$NH-),191($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{15}H_{12}N_2O_2S_2$; 316.39, found; 316(23.4, M^+).

4.7.2. 6-Acetyl-3-(4-chlorophenyl)-2-mercapto-5-methylthieno[2,3-d]pyrimidin-4(3H)-one (**16**).

Yield:70% ; mp:159°C; **IR** (KBr, ν, cm^{-1}): 3453 (NH amide), 1677 (C=O amide), 1217 (C=S) .**^{1}H NMR** (δ, ppm, $CDCl_3$): 2.6(s, 3H, -CO$\underline{CH_3}$), 2.8 (s, 3H, $\underline{CH_3}$-thiophene), 7.19 (d, 2H, Ar-H),7.5 (d, 2H, Ar-H). **^{13}C NMR** (δ, ppm, DMSO-d_6): 15 (CH_3 thiophene), 30 (CH_3 acetyl), 118, 135, 143,153 (thiophene quaternary carbons), 126, 129, 132,135 (phenyl), 163 (C-SH), 176 (-$\underline{CO}$NH-), 191($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{15}H_{11}ClN_2O_2S_2$; 350.84, found; 349.9(100, M^+).

4.7.3. 6-Acetyl-3-benzyl-2-mercapto-5-methylthieno[2,3-d]pyrimidin-4(3H)-one (**17**).

Yield:75% ; mp:211°C; **IR** (KBr,ν,cm^{-1}) : 3449 (NH amide), 1695 (C=O amide), 1295 (C=S).**^{1}H NMR** (δ, ppm, $CDCl_3$): 2.5(s, 3H, -CO$\underline{CH_3}$), 2.8 (s, 3H, $\underline{CH_3}$-thiophene), 5.7 (s, 2H, $\underline{CH_2}$-phenyl), 7.3-7.5 (m, 5H, Ar-H), 12.0 (s, 1H, -NH-). **^{13}C NMR** (δ, ppm, DMSO-d_6):15 (CH_3 thiophene), 30 (CH_3 acetyl), 117.9,135, 143,152 (thiophene quaternary carbons) ,128,128.5,129,136 (phenyl), 157 (C-SH) ,176 (-$\underline{CO}$NH-),191($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{16}H_{14}N_2O_2S_2$; 330.4, found; 329.9(100, M^+).

4.8.General procedure for synthesis of compounds (**18** and **19**).

A mixture of compound **15** or **17** (10 mmol) and hydrazine hydrate (5 mL) in pyridine(10 mL) was refluxed for 14 h .The reaction mixture was cooled, poured into crushed ice and the product was filtered and crystallized from ethanol.

4.8.1. 6-Acetyl-2-hydrazinyl-5-methyl-3-phenylthieno[2,3-d]pyrimidin-4(3H)-one (**18**).

Yield:39% ; mp: > 300 °C; **IR** (KBr, ν, cm^{-1}): 3447-3382 ($NHNH_2$).**^{1}H NMR** (δ, ppm, DMSO-d_6): 2.09 (s, 3H, -CO$\underline{CH_3}$), 2.4 (s, 3H, $\underline{CH_3}$-thiophene), 6.3-6.8 (br s, 2H, -NH$\underline{NH_2}$, D_2O exchangeable), 7.19 (d, 2H, Ar-H), 7.4 (d, 1H, Ar-H), 7.5 (m, 3H, 2 Ar-H + -$\underline{NH}NH_2$). **^{13}C NMR** (δ, ppm, DMSO-d_6): 15 (CH_3 thiophene), 30 (CH_3 acetyl), 118, 135, 143,153 (thiophene quaternary carbons), 126, 129 ,132, 135 (phenyl), 176 (-$\underline{CO}$NH), 191($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{15}H_{14}N_4O_2S$; 314.36, found; 314 (18, M^+).

4.8.2. 6-Acetyl-3-benzyl-2-hydrazinyl-5-methylthieno[2,3-d]pyrimidin-4(3H)-one (**19**).

Yield:30% ; mp: > 300 ˚C; **IR** (KBr,ν,cm^{-1}) :3387 ($NHNH_2$). **^{1}H NMR** (δ, ppm, DMSO-d_6): 2.5(s, 3H, -CO$\underline{CH_3}$), 2.8 (s, 3H, $\underline{CH_3}$-thiophene), 5.7 (s, 2H, $\underline{CH_2}$-phenyl), 7.3-7.5 (m, 5H, Ar-H).**^{13}C NMR** (δ, ppm, DMSO-d_6): 15 (CH_3 thiophene), 30 (CH_3 acetyl), 117.9,135, 143,152 (thiophene quaternary carbons), 128, 128.5, 129,136 (phenyl), 176 (-$\underline{CO}$NH-), 191($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{16}H_{16}N_4O_2S$; 328.39, found; 330 (21.5, M^+).

4.9.Synthesis of ethyl 5-acetyl-(((dimethylamino)methylene)amino)-4-methylthiophene-3-carboxylate (**20**).

A mixture of aminothiophene **1** (2.27gm,10 mmol) in DMFDMA (10 mL) was refluxed for 2h. The reaction mixture was cooled and the precipitate formed was filtered, washed and crystallized from ethanol.

Yield:53% ; mp: 99 ˚C; **^{1}H NMR** (δ, ppm, $CDCl_3$):1.3 (t, 3H, J=14.4, $\underline{CH_3}CH_2COO$), 2.4 (s, 3H, CH_3CO-), 2.6(s, 3H, CH_3-thiophene), 3.3 (s, 6H, $\underline{\text{dimethyl}}$ amino-), 4.3 (q, 2H, $CH_3\underline{CH_2}COO$-), 7.7 (s, 1H ,-N=$\underline{CH}$-).**^{13}C NMR** (δ, ppm, $CDCl_3$): 15 (CH_3 ester), 16.15 (CH_3 thiophene), 30 (CH_3 acetyl), 40 ($\underline{\text{dimethyl}}$ amino-), 61 ($CH_3\underline{CH_2}COO$-) , 111,127,143,153 (thiophene quaternary carbons), 164 (-N=$\underline{CH}$-), 171 (-$\underline{CO}$O-),191 ($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{13}H_{18}N_2O_3S$; 282.1, found; 282 (15.5, M^+).

4.10.Synthesis of ethyl 5-acetyl-2-formamido-4-methylthiophene-3-carboxylate(**21**).

$\underline{\text{Procedure A}}$:A mixture of compound **20** (2.5gm,10mmol) in glacial acetic acid (15 mL) was refluxed for 1h.The reaction mixture was cooled and poured into crushed ice. The precipitate was filtered and washed with water.

$\underline{\text{Procedure B}}$: A mixture of aminothiophene **1** (2.27 g, 10 mmol) and ammonium acetate (0.3 g, 3.89mmol) in formic acid (20 mL) was refluxed for 18 h .The reaction mixture was cooled and neutralized by diluted aqueous ammonia solution. The precipitate formed was filtered and crystallized from hexane.

Yield:63% ; mp: 107 ˚C; **^{1}H NMR** (δ, ppm, $CDCl_3$) :1.4 (t ,3H, J=14.4 ,$\underline{CH_3}CH_2COO$-) ,2.5 (s,3H,$\underline{CH_3}CO$-), 2.7 (s, 3H, CH_3-thiophene), 4.3 (q , 2H, $CH_3\underline{CH_2}COO$-), 8.6 (s, 1H ,-N$\underline{H}$CHO), 11.6 (s, 1H, -NHC$\underline{H}$O). **^{13}C NMR** (δ, ppm, $CDCl_3$) : 14.2 (CH_3 ester), 16

(CH_3 thiophene), 30 (CH_3 acetyl), 61 ($CH_3\underline{CH_2}COO$-), 115,127.4,143.5,149.7 (thiophene quaternary carbons),157 (-NH<u>C</u>HO) ,166 (-<u>CO</u>O-), 191 ($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{11}H_{13}NO_4S$;255.29, found; 254.9 (12.9, M^+).

4.11.Synthesis of 6-acetyl-1,5-dimethyl-2-(methylthio)-4-oxo-3-phenyl-3,4-dihydrothieno[2,3-d]pyrimidin-1-ium (**22**).

A mixture of compound **15** (3.16gm,10 mmol) in DMFDMA (20 mL) was refluxed for 3h.The reaction was cooled and poured into crushed ice. The yellow precipitate was filtered and crystallized from ethanol.

Yield: 30% ; mp:101°C; **^{1}H NMR** (δ, ppm,$CDCl_3$):1.27(s, 3H, -S-CH_3), 2.5(s, 3H,-$COCH_3$), 2.7 (s, 3H, CH_3-thiophene), 2.92 (s, 3H, -N-CH_3), 7.3 (m, 2H, Ar-H), 7.58 (m,3H,Ar-H). **^{13}C NMR** (δ, ppm, $CDCl_3$): 15.6 (-S-CH_3), 15.7(CH_3-thiophene), 30 (CH_3 acetyl), 30.6 (-N-CH_3) 128, 137,143 ,154 (thiophene quaternary carbons),120,129 ,130,135 (phenyl),162.3(C-SH) ,165(-<u>CO</u>NH-),191.8($CH_3\underline{CO}$-). **MS** m/z (%): calcd for $C_{17}H_{17}N_2O_2S_2$; 345.4, found; 345.9 (4.17, M^+).

4.12.General procedure for synthesis of compounds (**23**-**27**).

<u>Procedure A</u> : A mixture of appropriate aldehyde (10 mmol) and compound **15** (3.16gm, 10 mmol) was dissolved in (10 mL) absolute ethanol and stirred at room temperature for 15 minutes. Then 10 % NaOH solution was added dropwise to the reaction mixture. The mixture was stirred for 6 h, neutralized by 0.1 N HCl whereby the precipitation occurred. The precipitate formed was filtered, washed with water and crystallized from ethanol.

<u>Procedure B</u> : A mixture of appropriate aldehyde (10 mmol) and compound **15** (3.16gm, 10 mmol) was dissolved in 15 mL sodium ethoxide solution (0.2 g of sodium metal in 15 mL absolute ethanol) and stirred for 3h at room temperature. The precipitate formed was filtered and crystallized from ethanol. There was no significant difference between the two procedures in yield percentages.

4.12.1. 2-Mercapto-6-(3-(4-methoxyphenyl)acryloyl)-5-methyl-3-phenylthieno[2,3-d]pyrimidin-4(3H)-one (**23**).

Yield: 72% ; mp:278°C; **^{1}H NMR** (δ, ppm,$CDCl_3$): 2.9 (s, 3H, CH_3-thiophene), 3.8 (s, 3H, -O-CH_3), 6.9 (d, 2H, J=8, Ar-H), 7.1 (d, 1H, J=15.2, -CH=C<u>H</u>-CO-), 7.3 (d, 1H, Ar-H), 7.5-7.6 (m, 6H, Ar-H), 7.9 (d, 1H, J=15.2, -C<u>H</u>=CH-CO-). **^{13}C NMR** (δ, ppm,$CDCl_3$): 15.8(CH_3-thiophene), 55 (OCH_3)118.4, 127, 138.3,156 (thiophene quaternary carbons), 129.1, 145.5 (2 alkene carbons) 114.5, 120.6, 128.3 ,129.7, 130.6 (phenyl aromatic carbons), 162.2(C-SH) ,176(-<u>CO</u>NH-),183.8(-CH=CH<u>CO</u>-). **MS** m/z (%): calcd for $C_{23}H_{18}N_2O_3S_2$; 434.53, found; 434.04 (100, M^+).

4.12.2. 2-Mercapto-5-methyl-3-phenyl-6-(3-(p-tolyl)acryloyl)thieno[2,3-d]pyrimidin-4(3H)-one (**24**).

Yield: 59% ; mp:262°C; **^{1}H NMR** (δ, ppm, DMSO-d_6):2.3 (s, 3H, <u>CH_3</u>-thiophene), 2.8 (s, 3H, <u>CH_3</u> -Phenyl), 7 (d, 2H, J=7.6, Ar-H), 7.2 (t, 3H, J=13.6, Ar-H), 7.35 (m, 2H, Ar-H) , 7.4 (d, 1H, J=14.8, -CH=C<u>H</u>-CO-), 7.6 (d, 1H, J=15.2, -C<u>H</u>=CH-CO-), 7.7 (d, 2H, J=8, Ar-H).**^{13}C NMR** (δ, ppm, DMSO-d_6): 16.4(CH_3-thiophene), 21.5 (phenyl-<u>CH_3</u>) ,129.1 ,144.5 (2 alkene carbons)117.29,127, 132.3,142.4(thiophene quaternary carbons) ,124.5,126.1, 128.8 ,129.7 ,130 ,140.8 ,142.1(phenyl aromatic carbons), 161.8(C-SH) ,180.3(-<u>CO</u>NH-), 183.04(-CH=CH<u>CO</u>-). **MS** m/z (%): calcd for $C_{23}H_{18}N_2O_2S_2$; 418.53, found; 418.06(100, M^+).

4.12.3. 2-Mercapto-5-methyl-3-phenyl-6-(3-(3,4,5-trimethoxyphenyl)acryloyl)thieno[2,3-d]pyrimidin-4(3H)-one (**25**).

Yield: 76% ; mp:259°C; **^{1}H NMR** (δ, ppm, DMSO-d_6): 2.8 (s, 3H, <u>CH_3</u>-Thiophene), 3.7 (s, 3H, p-O<u>CH_3</u>), 3.8 (s, 6H, 2 m-OCH_3), 7 (d, 2H, J=8, Ar-H), 7.1 (br s, 2H, 2 H of trimethoxy phenyl ring), 7.2 (t, 1H, J=14.4, Ar-H), 7.3-7.4 (m, 3H, 2Ar-H & -CH=C<u>H</u>-CO-), 7.5 (d, 1H, J=15.6, -C<u>H</u>=CH-CO-).^{13}C NMR (δ, ppm, DMSO-d_6): 16.4(CH_3-thiophene), 56.5 (2 m-OCH_3), 60.6 (p-OCH_3) 129.7, 144.5 (2 alkene carbons) 106,130.7,139.9, 142(trimethoxy phenyl aromatic carbons) ,125.2 ,126 ,128.8 ,126 ,139 (phenyl aromatic carbons), 117,126.9,142.6 , 153.5 (thiophene quaternary aromatic carbons), 161(C-SH), 168.8(-<u>CO</u>NH-), 183.04(-CH=CH<u>CO</u>-). **MS** m/z (%):calcd for $C_{25}H_{22}N_2O_5S_2$; 494.58, found; 494.6(48.2, M^+).

4.12.4. 2-Mercapto-5-methyl-3-phenyl-6-(3-(thiophen-2-yl)acryloyl)thieno[2,3-d]pyrimidin-4(3H)-one (**26**).

Yield: 66% ; mp272°C; ^{1}H NMR (δ, ppm,$CDCl_3$)**:** 2.9 (s, 3H, CH_3-thiophene),7.1 (d, 2H, J=14.8, Ar-H), 7.4 (d, 1H, -CH=CH-CO-), 7.5-7.6 (m, 6H, Ar-H), 8 (d, 1H, J=15.2,-CH=CH-CO-).**^{13}C NMR** (δ, ppm,$CDCl_3$): 15.7(CH_3-thiophene), 117,128, 137,145(thiophene quaternary carbons),129.1,143.13 (2 alkene carbons)121.9, 128.3 ,139.8 ,128.5 (phenyl aromatic carbons) ,139.8,129.7,132.9,128.3(thiophene carbons) 162(C-SH), 163(-CONH-),173(-CH=CHCO-). **MS** m/z (%): calcd for $C_{20}H_{14}N_2O_2S_3$; 410.02, found; 409.97(100, M^+).

4.12.5. 6-(3-(4-Bromothiophen-2-yl)acryloyl)-2-mercapto-5-methyl-3-phenylthieno[2,3-d]pyrimidin-4(3H)-one (**27**).

Yield: 46% ; >300 mp°C ; **^{1}H NMR** (δ, ppm, DMSO-d_6): 2.1 (s, 3H, CH_3-thiophene), 7.1 (d, 1H, J=11.6, -CH=CH-CO-), 7.3-7.5 (m, 7H, Ar-H), 7.8(d, 1H, J=15.2, -CH=CH-CO-). **^{13}C NMR** (δ, ppm,DMSO-d_6): 15 (CH_3-thiophene), 117,129.6, 134,141.7 (thiophene quaternary carbons) ,129.1,143.13 (2 alkene carbons)129.3 (phenyl aromatic carbons) ,86,128.3,130.5,132(bromo thiophene carbons) 162(C-SH) ,163 (-CONH-),173(-CH=CHCO-). **MS** m/z (%): calcd for $C_{20}H_{13}BrN_2O_2S_3$; 489.42, found; 489.3(100, M^+).

4.13.Target prediction

The target compounds **2-27** were uploaded in Tripos Mol2 format. PharmMapper finds the best mapping poses of the uploaded molecule against all the targets in Pharm Target DB and top N potential drug targets as well as respective molecule's aligned poses are outputted.

4.14.*In vitro* antitumor screening

Materials and methods

Five human tumor cell line namely ; hepatocellular carcinoma (HepG-2) , Mammary gland (MCF-7), epitheliod Carcinoma (HeLa) , epidermoid carcinoma (Hep2) and Human prostate cancer (PC-3) were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. Doxorubicin was used as a

standard anticancer drug for comparison. The reagents used were RPMI-1640 medium , MTT and DMSO (sigma co., St. Louis, USA), Fetal Bovine serum (GIBCO, UK).

MTT procedure

The cell lines mentioned above were used to determine the inhibitory effects of compounds on cell growth using the MTT assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100µg/ml streptomycin at 37 C in a 5% Co_2 incubator. The cell lines were seeds in a 96-well plate at a density of $1.0x10^4$ cells/well. at 37 C for 48 h under 5% Co_2. After incubation the cells were treated with different concentration of compounds and incubated for 24 h. After 24 h of drug treatment, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570 nm using a plate reader (EXL 800 , USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X 100. The IC_{50} values were calculated according to the equation for Boltzmann sigmoidal concentration response curve using the nonlinear regression fitting models.

4.15.DNA/methyl green assay

DNA methyl green (20 mg) was suspended in 100 ml of 0.05 MTris-HCl buffer (pH 7.5) containing 7.5 mM $MgSO_4$; the mixture was stirred at 37 °C with a magnetic stirrer for 24 h. Test samples(10, 100, 1000 mg) were dissolved in ethanol in ependoff tubes, solvent was removed under vacuum, and 200 mL of the DNA/methyl green solution were added to each tube. Samples were incubated in the dark at ambient temperature. After 24 h, the final absorbance of the samples was determined at 642.5–645 nm. Readings were corrected for initial absorbance and normalized as the as the percentage of the untreated standard using ethidium bromide as positive control.

4.16.Enzyme inhibition assay

The *in vitro* ability of test compounds to inhibit DNA polymerase, thymidylate synthase and tyrosine kinase was carried out using human taq DNA polymerase beta kit (catalog NO. 9001-500,Biovision,USA), human thymidylate synthase(TS) ELISA kit (Cat No. MBS261554, MyBiosource.com) and protein tyrosine kinase assay Kit, non-radioactive (Catalog Number PTK101,Sigma Aldrich, USA) according to the manufacturer's instructions. The tested compounds were dissolved in DMSO and serially diluted into different concentrations.

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Highlites

- New thiophene(**2-13**) and thienopyrimidine (**15-27**) derivatives have been synthesized. Twenty three compounds were screened against five cell lines namely; hepatocellular carcinoma (liver) HePG-2, epdermoid carcinoma (larynx) HEP-2, mammary gland (breast) MCF-7, human prostate cancer PC-3 and epitheloid cervix carcinoma Hela.
- The results revealed that compounds **15**,**16**,**17**,**24** and **25** showed the highest antitumor activity against all tested cell lines compared to Doxorubicin.
- In order to explain the mode of action of the observed anticancer activity, compounds **15**,**16**,**17**,**24** and **25** were selected to screen their DNA binding affinity and enzyme inhibitory activity against DNA polymerase, thymidylate synthase and tyrosine kinase. The results revealed that the tested compounds showed good DNA binding affinity as well as good inhibitory activity against the three enzymes which might explain the observed anticancer activity of the target compounds.
- compound **17** was identified as the most potent inhibitor for the three tested enzymes with IC_{50} 0.9, 27.9 and 73.4 µM respectively.
- Also, compounds **16**,**24** and **25** showed high enzyme inhibition percentages against the screened enzymes So, this study raveled that compounds **16,17,24 and 25** could be promising leads for further development of more potent antitumor agents.

Graphical abstract