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Design, Synthesis and Pharmacological Evaluation of Novel Annelated Pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4]Triazines as Antimicrobial Agents

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Abstract: In our tactic to construct bio-active molecules, a series of novel pyrimido[2,1-*c*][1,2,4]triazine-3,4-diones based heterocycles, were synthesized and evaluated for their *in vitro* antimicrobial impacts. The exploratory bioassay results declared that, the majority of the evaluated compounds exhibited considerable anti-microbial activity comparable to the reference drugs. Conjugates **15j**, **15f**, **15i**, **15h**, **15g** and **15a** were found to be the most potent antibacterial, indicating that conjugates bearing electron-attracting substituents exhibited higher potency than these with electron-releasing substituents.

Keywords: Mannich reaction, 1,2,4-triazine, pyrimidine, 1,2,4-triazole, *N*-heterocycles, anti-microbial activity

1. Introduction

The vast use of antibiotics in the 1940s, puts a limit of the women's death from post-childbirth infections, and diseases such as tuberculosis were rife, using of antibiotics provide us the ability to avoid these tragedies. But so, micro-organisms become resistant to antimicrobial treatments, including antibiotics, there is a very real possibility that the drugs we have come to rely upon may become obsolete. A potential solution to counteract antibiotic resistance is to design and explore innovative heterocyclic scaffolds with novel modes of action. Thus, vast attention is given by medicinal chemists to 1,2,4-triazoles, pyrimidines and triazines motifs for developing of many drugs with various biological activities. Due to their ability to bind easily with enzymes and receptors in organisms, *via* H-bonding and other weak interactions, azoles and/or azines have been attracting increasing interest in pharmaceutical chemistry [1,2]. Potent pharmacological activity of azoles and/or azines can attributed to the presence of toxophoric azomethine moiety ($-C=N-$), that used in forming H-bonding with nucleophilic centers of various cellular constituents, thus upsetting the normal metabolic operation of these organisms [3].

1,2,4-Triazine analogues are the first heteroaryl in this prelude are a part of a large amount of natural and synthetic biologically active compounds, have got their origin from natural and synthetic sources as exemplified by Azaribine, Triazavirin, Metribuzin and Lamotrigine (**Figure 1**), which have important biological activities as antiviral drugs [4]. Tumor growth inhibitors [5-8], antiviral [9], antibacterial, and antifungal [8,10].

Triazoles, in particular 1,2,4-triazole, the second motif in this prelude, possess wide spectrum of biological activities, including antibacterial activity, the chemical manipulations of the triazole moiety results in the improvement of highly effective analogues that have improved selectivity [11]. Recent studies have reported the remarkable anti-cancer activity of recently synthesized 1,2,4-triazoles towards many tumor cell lines [12-14]. A vast number of 1,2,4-triazole derivatives has been reported with significant antifungal, antibacterial, antiviral, anticonvulsant, antituberculosis, antidepressant, anti-inflammatory and anticancer activities [15-17].

Finally, as third motif in this preface, pyrimidines have a long and eminent history extending from the days of their discovery as important constituents of nucleic acids to their current use in the chemotherapy of AIDS. Alloxan is known with its diabetogenic action in a number of animals [18]. Uracil, thymine and cytosine (**Figure 1**), are the three important constituents of nucleic acids, pyrimidine derivatives. In addition, the pyrimidine ring is found in vitamins like barbitone [18], the first barbiturate hypnotic sedative and anticonvulsant is a pyrimidine derivative [18]. 5-Fluorouracil is a pyrimidine derivative, that is the early metabolites prepared [19], also, 5-Thiouracil derivatives exhibits some useful antineoplastic activities [20]. On the basis of the above considerations about the

chemistry as well as the biological significance of triazole, pyrimidine and triazine analogues, and to continue our previously initiated program [20,39], aiming at merge of multi pharmacophores in single architecture, to study and develop a novel innovative route for synthesis of various heterocyclic systems of promising biological activities. Herein, we describe the synthesis and the utility of 8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (**2**), as reactive precursor to attain a series of the target compounds.

Figure 1. Representative examples of bioactive 1,2,4-triazole, 1,2,4-triazine and pyrimidine derivatives

2. Results and Discussion

2.1. Chemistry

Our starting point is to prepare 8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4] triazine-3,4-dione (**2**), as a versatile precursor for annulation the target novel *N*-heterocyclic systems, to evolve probes of admirable therapeutic potentials. So, treatment of the hydrazonyl analogue **1** [35,36], with oxalyl chloride in refluxing DMF containing Et₃N as catalyst, afforded the 1,2-dioxo-triazine analogue **2**. The cyclic 1,2-bioxygen analogues are well known to be reactive, and were utilized as potential precursors for building various heterocyclic systems, that can possess

awaited biological activity. Thus, treatment of compound **2** with thiosemicarbazide in refluxing EtOH/HCl, afforded 2-(4-oxo-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazin-3(4*H*)-ylidene)hydrazinecarbothioamide (**3**) in 80% yield, (**Scheme 1**, **Tables 1** and **2**). This reaction can lead to three possible structures (**3**, **4** and **5**), these could be formed if the geometry of the thiophene moiety in compound **2** is out of plane so, the two 1,2-dioxo are in suitable situation for incorporating in cyclocondensation reactions, with the two amino groups of thiosemicarbazide, but herein the steric hindrance has a dramatic effect in orientation the reaction. Therefore, structures (**4** and **5**) were ruled out, with consideration the structure **3** as the reaction product. That is also supported by spectral data and elemental analyses; the presence of the thiocarbonyl group is attested by ^{13}C NMR (singlet at 181.2 ppm), and IR (absorption band at 1290 cm^{-1}). Furthermore, the recorded mass at m/z 397.08 corresponds to the formula $\text{C}_{17}\text{H}_{15}\text{N}_7\text{OS}_2$ all support the formation of the thiosemicarbazone derivative **3**, via simple nucleophilic substitution of one amino group to afford thioureido analogue **3**, without further cyclocondensation into **4** or **5**.

Scheme 1. Reagent, conditions and synthesis of compounds (**2** and **3**)

Chlorination of the 1,2-dioxo compound **2** with POCl₃, affording 3-chloro-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-4*H*-pyrimido[2,1-*c*][1,2,4]triazin-4-one (**6**) in 75% yield. The ¹H NMR as well as IR spectra of compound **6** confirmed the absence of N-H group, while, its ¹³C NMR spectrum showed a singlet at 149.1 ppm attributed to C-Cl, (Scheme 2). The chloro triazine **6** underwent further, hydrazinolysis with excess of N₂HNH₂.H₂O to afford the hydrazinyltriazine derivative **7**, which undergoes further cyclization aiming at exploring the reactivity of its hydrazinyl tag, in building triazolotriazine derivatives. Thus, the hydrazinyl **7** was underwent to various one carbon donors, to afford the target azoles and/or azines (**8**, **10-12**), (Scheme 2). The structures of isolated products (**8**, **10-12**), were confirmed *via* spectral data together with correct elemental analyses (Tables 1 and 2). Signals of the hydrazinyl protons, originally observed in **7** (¹H NMR) at 4.82 and 8.32 ppm, were disappeared in each case. The mass spectrum of compound **12** showed a peak at *m/z* 396.06, corresponding to its molecular formula C₁₈H₁₃ClN₆OS. As a chemical confirmation the triazolotriazine compound **10**, was obtained in another design *via* treating the hydrazinyl **7** with dimethylformamide-dimethyl acetal (DMF-DMA) in refluxing dry xylene, but in low yield 60% (Scheme 2).

On the other hand, the reactivity of the endocyclic imino group in compound **8** was investigated. Thus, hydroxymethylation of **8** *via* refluxing with MeOH/CH₂O solution, afforded 2-(hydroxymethyl)-4,8-diphenyl-10-(thiophen-2-yl)-9,10-dihydropyrimido[2,1-*c*][1,2,4]triazino[3,4-*f*][1,2,4]triazin-12(2*H*)-one (**9**) in 75% yield (Scheme 2). The structure of compound **9** was deduced based on its spectral data, where, its mass spectrum recorded molecular ion peak (C₂₅H₂₀N₆O₂S) at *m/z* 468.15, while the IR spectrum showed characteristic absorption bands at 3315 cm⁻¹ due to stretching of the O-H group. The ¹H NMR spectrum displayed D₂O exchangeable broad singlet at 5.19 ppm for the O-H group, besides singlet signal at 4.32 ppm attributed to methylene protons of hydroxy methyl moiety, (Tables 1 and 2).

Scheme 2. Reagent, conditions and synthesis of compounds (**6-12**)

On the other hand, aiming at annulation a new Mannich analogues to investigate their biological impact, the thiol tautomer **14** was synthesized, then the reactivity of its thiol tag was investigated *via* subjection to one-pot three component reaction with various substituted anilines and excess of HCHO solution, in EtOH to give the new *N*-Mannich derivatives (**15a-j**), in good to excellent yields (70-80%), (**Scheme 3**). Formation of the compounds (**15a-j**), can be demonstrated on the basis of initial Mannich reaction which proceeds *via* two steps, first the reaction between HCHO and the amine leads to the formation of the non-isolable iminium ion intermediate, which lose H_2O molecule *in situ*. Secondly, the thiocarbonyl compound undergoes tautomerization to give its thiol tautomer, which proceeds to execute an attack on the iminium ion, this attack finally yields the target β -amino-carbonyl compounds (**15a-j**) as shown in Scheme 4. The IR spectra of the isolated compounds (**15a-j**) displayed the common characteristic absorption bands around the region $3165\text{-}3281\text{ cm}^{-1}$ due to secondary amine groups. Further, evidence for compounds (**15a-j**) is based on their ^1H NMR spectra; the secondary amine proton appeared at $\approx 4.80\text{ ppm}$ as a singlet (D_2O exchangeable), while the methylene singlet (^1H

NMR) of their phenylaminomethyl moiety were observed at ≈ 5.40 ppm. The IR spectrum of **15j** showed characteristic absorption bands at 1390 and 1520 cm^{-1} due to $\text{NO}_{2\text{str}}$ (Asy. & Sy.), respectively. Its mass spectrum displayed an ion peak at m/z 530.09 (M^+ , 30%) corresponding to the expected molecular formula $\text{C}_{24}\text{H}_{18}\text{N}_8\text{O}_3\text{S}_2$, (Tables 1 and 2).

Scheme 3. Reagent, conditions and synthesis of compounds (**14** and **15**)

Scheme 4. The proposed mechanism for the formation of compounds **15**.

Compound **7** was also served as a facile point to departure to other pyrimido-[1,2,4]triazolo-[1,2,4]triazine derivatives, *via* interaction with some other one carbon donor reagents. Thus, compound **7** cyclocondensed smoothly with KSCN, ethyl cyanoacetate, acetic anhydride, benzoyl chloride and thionyl chloride to afford pyrimido-[1,2,4]triazolo-[1,2,4]triazine derivatives (**5**, **16-19**) (Scheme 5). The reaction of hydrazinyl derivative **7** with KSCN was assumed to proceed *via* formation of the non-isolable intermediates which, undergo further intramolecular cyclization *via* loss of H_2S molecule to afford the final product **5**, as shown in the considered mechanism (Scheme 6). The spectral data together with elemental analyses of the isolated compounds (**5**, **16-19**), were in covenant with the

suggested structures. The ^1H NMR spectra of these compounds showed the lack of the signals of the hydrazinyl protons, originally observed in **7** (^1H NMR) at 4.82 and 8.32 ppm. A recorded m/z value of 387.08, supported the formation of compound **16**, while, its IR spectrum declared strong absorption bands at 1686 and 2218 cm^{-1} due to $\text{C}=\text{O}$ and $\text{C}\equiv\text{N}$ groups, respectively.

Scheme 5. Reagent, conditions and synthesis of compounds (**5**, **16-19**), i = KSCN /AcOH; ii = $\text{CNCH}_2\text{CO}_2\text{Et}$; iii = Ac_2O ; iv = PhCOCl ; v = $\text{SOCl}_2/\text{EtOH}$

Scheme 6. The proposed mechanism for formation of compound **5**.

Furthermore, the reactivity of the endocyclic imino moiety in compound **2**, was explored aiming at annulation of pendent *N*-pyrimido[*c*]pyridazine architectures. Thus, subsequent chloroacetylation of **2** in warm EtOH containing catalytic amount of Et_3N , gave **20** in 80% yield. The recorded mass at m/z 400.05 corresponding to the formula $\text{C}_{18}\text{H}_{13}\text{ClN}_4\text{O}_3\text{S}$, besides the CH_2 singlet (^1H NMR) at 4.21 ppm

and its corresponding (^{13}C NMR) signal at 42.4 ppm all favored formation of **20**, (**Scheme 7**). The latter compound **20** was incorporated in a sequence of manipulations, aiming at exploiting the reactivity of its chloroacetyl tag to annulate the target *N*-pyrimido[*c*]pyridazine. Thus, hydrazinolysis of compound **20** with $\text{H}_2\text{NNH}_2 \cdot \text{H}_2\text{O}$ afforded the corresponding hydrazniyl derivative **21**, which, underwent further, cyclization *via* treating with malononitrile to afford *o*-aminocarbonitriles **22**. Heating of compound **22** in acetic anhydride/pyridine mixture, gave 2-(5-hydroxy-7-methyl-2,3-dihydropyrimido[4,5-*c*]pyridazin-4-yl)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (**23**), (**Scheme 7**). The IR spectrum of **23** revealed the presence of O-H stretching absorption band at 3453cm^{-1} , N-H stretching absorption band at 3251cm^{-1} , two amidic C=O stretching bands at 1662 and 1679cm^{-1} . Its ^1H NMR spectrum displayed two broad singlets at 6.92 and 9.31 ppm for the NH and OH groups, respectively. The mass spectrum of **23** displayed an intense peak at m/z 486.12 (M^+ , 55%) corresponding to the expected molecular formula $\text{C}_{23}\text{H}_{18}\text{N}_8\text{O}_3\text{S}$.

Scheme 7. Reagent, conditions and synthesis of compounds **20-23**

In another investigation the chloroacetyl moiety in compound **20**, was cyclized to annulate the thiazole analogues, *via* easily condensation with thiourea in boiling $\text{EtOH}/\text{K}_2\text{CO}_3$ solution, to afford the non-isolable intermediates **24-26**, which, underwent further intramolecular cyclization afforded 2-

aminothiazole derivative **27**, (**Scheme 8**). Likewise, compound **20** was reacted with NH_4SCN in refluxing EtOH to afford the intermediate **28**, *via* nucleophilic substitution of the chlorine atom by the thiocyanate moiety, subsequent intramolecular cyclization *via* nucleophilic addition of the OH to the nitrile group, afforded thiazole analogue **30**, in 76% yield. A recorded m/z value of 423.03, besides the most characteristic signal (^1H NMR) of compound **30**, belong to thiazole exchangeable (N-H) proton at 10.13 ppm, all support the formation of compound **30**.

Scheme 8. Reagent, conditions and synthesis of compounds **27-30**

On the other hand, the thioureido analogue **3** was incorporated in a sequence of manipulations, aiming at investigation the reactivity of its thioureido moiety to attain the target thiazole and/or thiazine architectures. Thus, compound **3** was treated with benzylidenemalononitrile in boiling dioxane, to furnish 1,3-thiazine-5-carbonitrile derivative **32**, *via* the intermediate **31**, which undergoes intramolecular cyclization *via* nucleophilic addition of the NH₂ to the nitrile group, afforded 1,3-thiazine analogue **32**, in 75% yield, (**Scheme 9**). The IR spectrum of **32** revealed the presence of intense absorption bands at 3325 and 2219 cm⁻¹ attributed to NH₂_{str.} and C≡N_{str.} groups, respectively. Furthermore, thioureido **3** was reacted with 3-chloropentane-2,4-dione in refluxing EtOH to afford 2-substituted 4-methyl-5-acetylthiazole derivative **33**. Similarly, compound **3** was cyclized in another investigation with DMAD in refluxing dioxane, aiming at annulation of thiazine analogue **35**, (**Scheme 9**). However, the recorded m/z at 507.08 of the isolated product, ruled out this assumption,

and supported the formation of the thiazole derivative **35**, in 80% yield, *via* formation the intermediate **34** which *in situ* cyclized through loss of MeOH molecule, as shown in Scheme 9. Formation of compound **35** can be explained on the base of initial Michael type addition of the thiol function in thioureido moiety to the activated triple bond in DMAD to afford the non-isolable intermediate **34**, which, undergoes intramolecular cyclization *via* loss of another MeOH molecule (route a), to yield the thiazole derivative **35**. The IR spectra of these compounds showed the lack of carbothioamide moiety absorption bands originally observed in **3**, (IR) at 1290 and 3230 cm^{-1} ppm. The most characteristic signal of compound **33** (^1H NMR), due to the thiazole exchangeable (N-H) proton at 10.83 ppm, in addition of two new singlets were observed at 2.51 and 2.72 ppm, attributed to the methyl and acetyl protons, respectively, all confirmed the structure of compound **33**.

Scheme 9. Reagent, conditions and synthesis of compounds **32** and **35**

Table 1. Physical and analytical data of compounds **2** to **35**

Comp.	mp °C	Yield %	Mol formula (Mol. Wt)	Recrystallizing solvent	Microanalysis Calc. (Found) %		
					C	H	N
2	252-254	75	C ₁₆ H ₁₂ N ₄ O ₂ S (324.07)	EtOH	59.25(59.05)	3.73(3.12)	17.27(17.11)
3	271-273	80	C ₁₇ H ₁₅ N ₇ OS ₂ (397.08)	EtOH	51.37(51.10)	3.80(3.19)	24.67(24.32)
5	205-207	76	C ₁₇ H ₁₃ N ₇ OS (363.09)	EtOH	56.19(56.01)	3.61(3.51)	26.98(26.98)
6	176-178	75	C ₁₆ H ₁₁ ClN ₄ OS (342.03)	MeOH-H ₂ O	56.06(55.98)	3.23(3.01)	16.34(16.20)
7	220-222	80	C ₁₆ H ₁₄ N ₆ OS (338.09)	EtOH	56.79(56.51)	4.17(4.01)	24.84(24.63)
8	242-244	78	C ₂₄ H ₁₈ N ₆ OS (438.13)	EtOH	65.74(65.50)	4.14(4.07)	19.17(19.03)
9	182-184	75	C ₂₅ H ₂₀ N ₆ O ₂ S (468.15)	DMF	64.09(64.11)	4.30(4.20)	17.94(17.59)
10	212-214	60	C ₁₇ H ₁₂ N ₆ OS (348.08)	EtOH	58.61(58.32)	3.47(3.12)	24.12(24.01)
11	257-259	65	C ₁₇ H ₁₂ N ₆ O ₂ S (364.07)	MeOH	56.04(56.11)	3.32(3.05)	23.06(23.1)
12	260-262	76	C ₁₈ H ₁₃ ClN ₆ OS (396.06)	EtOH	54.48(54.37)	3.30(3.11)	21.18(21.04)
14	232-234	78	C ₁₇ H ₁₂ N ₆ OS ₂ (380.05)	EtOH	53.67(53.38)	3.18(3.11)	22.09(22.01)
15a	218-220	72	C ₂₄ H ₁₉ N ₇ OS ₂ (485.11)	MeOH	59.36(59.14)	3.94(3.78)	20.19(20.06)
15b	202-204	79	C ₂₅ H ₂₁ N ₇ OS ₂ (499.12)	Acetone	60.10(60.01)	4.24(4.17)	19.62(19.51)
15c	165-167	75	C ₂₅ H ₂₁ N ₇ OS ₂ (499.12)	MeOH	60.10(59.81)	4.24(4.03)	19.62(19.43)
15d	196-198	70	C ₂₅ H ₂₁ N ₇ O ₂ S ₂ (515.12)	EtOH	58.24(58.11)	4.11(3.98)	19.02(18.95)

Table 1 continued

Comp.	mp °C	Yield %	Mol formula (Mol. Wt)	Recrystallizing solvent	Microanalysis Calc. (Found)		
					C	H	N
15e	210-212	80	C ₂₅ H ₂₁ N ₇ O ₂ S ₂ (515.12)	EtOH	58.24(58.04)	4.11(4.01)	19.02(18.86)
15f	267-269	75	C ₂₄ H ₁₈ FN ₇ OS ₂ (503.10)	Acetone	57.24(57.13)	3.60(3.36)	19.47(19.25)
15g	138-140	77	C ₂₄ H ₁₈ ClN ₇ OS ₂ (519.07)	DMF-MeOH (1:3)	55.43(55.19)	3.49(3.24)	18.85(18.63)
15h	203-205	71	C ₂₄ H ₁₈ N ₈ O ₃ S ₂ (530.09)	EtOH	54.33(54.24)	3.42(3.19)	21.12(21.01)
15i	154-456	73	C ₂₄ H ₁₈ N ₈ O ₃ S ₂ (530.09)	EtOH	54.33(54.15)	3.42(3.10)	21.12(21.05)
15j	281-283	78	C ₂₄ H ₁₈ N ₈ O ₃ S ₂ (530.09)	MeOH-H ₂ O	54.33(54.18)	3.42(3.25)	21.12(20.98)
16	178-180	65	C ₁₉ H ₁₃ N ₇ OS (387.08)	EtOH	58.90(58.71)	3.38(3.15)	25.31(25.14)
17	156-168	60	C ₁₈ H ₁₄ N ₆ OS (362.09)	CHCl ₃	59.65(59.47)	3.89(3.62)	23.19(23.07)
18	275-277	76	C ₂₃ H ₁₆ N ₆ OS (424.11)	MeOH	65.08(65.01)	3.80(3.71)	19.80(19.51)
19	231-233	65	C ₁₆ H ₁₂ N ₆ O ₂ S ₂ (384.05)	EtOH	49.99(49.78)	3.15(3.02)	21.86(21.75)
20	128-130	80	C ₁₈ H ₁₃ ClN ₄ O ₃ S (400.05)	EtOH/dioxane (2:1)	53.94(53.76)	3.27(3.11)	13.98(13.19)
21	227-229	76	C ₁₈ H ₁₆ N ₆ O ₃ S (396.10)	EtOH	54.54(54.36)	4.07(4.01)	21.20(21.11)
22	221-223	75	C ₂₁ H ₁₆ N ₈ O ₂ S (444.10)	CHCl ₃	56.75(56.54)	3.63(3.41)	25.21(25.12)
23	240-242	76	C ₂₃ H ₁₈ N ₈ O ₃ S (486.12)	EtOH	56.78(56.50)	3.73(3.61)	23.03(23.01)
27	231-233	80	C ₁₉ H ₁₄ N ₆ O ₂ S ₂ (422.06)	DMF-MeOH (1:3)	54.01(54.12)	3.34(3.11)	19.89(19.57)
30	189-191	76	C ₁₉ H ₁₃ N ₅ O ₃ S ₂ (423.03)	EtOH	53.89(53.68)	3.09(3.01)	16.54(16.28)

Table 1 continued

Comp.	mp °C	Yield %	Mol formula (Mol. Wt)	Recrystallizing solvent	Microanalysis Calc. (Found)		
					C	H	N
32	165-167	75	C ₂₇ H ₁₉ N ₉ OS ₂ (549.12)	DMF	59.00(58.85)	3.48(3.25)	22.94(22.67)
33	145-147	65	C ₂₂ H ₁₉ N ₇ O ₂ S ₂ (477.09)	EtOH	55.33(55.19)	4.01(4.03)	20.53(20.39)
35	228-230	80	C ₂₂ H ₁₇ N ₇ O ₄ S ₂ (507.08)	MeOH-H ₂ O	52.06(51.98)	3.38(3.15)	19.32(19.18)

Table 2. Spectral data of compounds 1-35

Comp.	IR (KBr) ν max in cm ⁻¹	¹ H-NMR (DMSO- <i>d</i> ₆) in ppm	¹³ C-NMR (DMSO- <i>d</i> ₆) in ppm
2	689 (C-S-C), 1610-1620 (2 C=N), 1645-1685 (2 amidic C=O), 3235 (N-H).	2.82 (dd, <i>J</i> = 13.7 and 4.7 Hz, 1H), 2.91 (dd, <i>J</i> = 9.5 and 13.7 Hz, 1H), 5.09 (dd, <i>J</i> = 9.5 and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.05 (d, <i>J</i> = 4.5, 1H, Thioph. _(C5) -H), 10.56 (s, 1H, NH _{Deutr.} Exch).	
3	685 (C-S-C), 1290 (C=S), 1610-1620 (3 C=N), 1646 (amidic C=O), 3410–3230 (2 NH and NH ₂).	2.81 (dd, <i>J</i> = 13.7 and 4.7 Hz, 1H), 2.90 (dd, <i>J</i> = 9.5 and 13.7 Hz, 1H), 5.10 (dd, <i>J</i> = 9.5 and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.21 (d, <i>J</i> = 4.5, 1H, Thioph. _(C5) -H), 8.56 (s, 2H, NH ₂ Deutr. Exch), 10.59 (s, 1H, NH _{Deutr.} Exch).	41.0 (CH ₂), 51.9 (CH), 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 131.1, 140.5 (C-Ar), 144.6, 149.1, 164.5 (3 C=N-), 160.4 (C=O), 181.2 (C=S).
5	683 (C-S-C), 1613-1625 (4 C=N), 1650 (amidic C=O), 3340 (NH ₂).	2.83 (dd, <i>J</i> = 13.7 and 4.7 Hz, 1H), 2.91 (dd, <i>J</i> = 9.5 and 13.7 Hz, 1H), 5.10 (dd, <i>J</i> = 9.5 and 3.9 Hz, 1H), 6.86 (s, 2H, NH ₂ Deutr. Exch), 7.69–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.21 (d, <i>J</i> = 4.4, 1H, Thioph. _(C5) -H).	

Table 2 continued

Comp.	IR (KBr) ν max in cm^{-1}	^1H -NMR (DMSO- d_6) in ppm	^{13}C -NMR (DMSO- d_6) in ppm
6	685 (C-Cl), 687 (C-S-C), 1610-1620 (3 C=N), 1645 (amidic C=O).	2.83 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.91 (dd, $J = 9.5$ and 13.7 Hz, 1H), 5.14 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.25 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	41.0 (CH ₂), 51.8 (CH), 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 131.1, 140.5 (C-Ar), 144.6, 164.5 (2 C=N-), 149.1, (=C-Cl), 160.3 (C=O), 181.2 (C=S).
7	687 (C-S-C), 1610-1620 (3 C=N), 1645 (amidic C=O), 3211-3351 (NH, NH ₂).	2.85 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.91 (dd, $J = 9.5$ and 13.7 Hz, 1H), 4.82 (s, 2H, NH ₂ Deutr. Exch), 5.14 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.25 (d, $J = 4.6$, 1H, Thioph. _(C5) -H), 8.32 (s, 1H, NH ₂ Deutr. Exch).	
8	689 (C-S-C), 1611-1624 (3 C=N), 1648 (amidic C=O), 3231(NH).	2.81 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.90 (dd, $J = 9.5$ and 13.7 Hz, 1H), 4.94 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 13H, Ar-H, Triazin. _(C6) -H and Thioph. _(C3,4) -2H, 8.25 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 8.32 (s, 1H, NH ₂ Deutr. Exch).	
9	685 (C-S-C), 1609-1621(3 C=N), 1645 (amidic C=O), 3315(OH).	2.83 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.92 (dd, $J = 9.5$ and 13.7 Hz, 1H), 4.94 (dd, $J = 9.5$ and 3.9 Hz, 1H), 4.32 (s, 2H, CH ₂ -OH), 5.19 (s, 1H, OH ₂ Deutr. Exch), 7.67–7.78 (m, 13H, Ar-H, Triazin. _(C6) -H and Thioph. _(C3,4) -2H), 8.25 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	
10	687 (C-S-C), 1610-1620 (4 C=N), 1645 (amidic C=O).	2.81 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.89 (dd, $J = 9.2$ and 13.5 Hz, 1H), 5.14 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.25 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 9.52 (s, 1H, Triazol. _(C3) -H).	32.1, 40.1 (2CH ₂), 51.8 (CH), 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 131.1, 140.5 (C-Ar), 153.5, 157.6, 160.5, 164.5 (4 C=N-), 160.3 (C=O).
11	687 (C-S-C), 1610-1620 (3 C=N), 1642-1655 (2 amidic C=O), 3210 (NH).	2.80 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.88 (dd, $J = 9.5$ and 13.7 Hz, 1H), 5.13 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.65–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.25 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 9.41 (s, 1H, NH ₂ Deutr. Exch).	

Table 2 continued

Comp.	IR (KBr) ν max in cm^{-1}	$^1\text{H-NMR}$ ($\text{DMSO-}d_6$) in ppm	$^{13}\text{C-NMR}$ ($\text{DMSO-}d_6$) in ppm
12	659 (C-Cl), 687 (C-S-C), 1610-1620 (4 C=N), 1645 (amidic C=O).	2.82 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.90 (dd, $J = 9.2$ and 13.5 Hz, 1H), 4.23 (s, 2H, CH ₂), 5.16 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.20 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	
14	687 (C-S-C), 1610-1620 (4 C=N), 1641 (amidic C=O), 2580 (S-H).	2.81 (dd, $J = 13.5$ and 4.3 Hz, 1H), 2.90 (dd, $J = 9.3$ and 13.6 Hz, 1H), 5.14 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.62–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.18 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 11.52 (s, 1H, SH _{Deutr. Exch.}).	32.1, 40.1 (2CH ₂), 51.8 (CH), 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 131.1, 140.5 (C-Ar), 153.5, 157.6, 164.5 (3 C=N-), 160.3 (C=O), 167.9 (N=C-SH).
15a	687 (C-S-C), 1609-1624 (3 C=N), 1643 (amidic C=O), 1281 C=S), 3219 (N-H).	2.79 (dd, $J = 12.9$ and 4.2 Hz, 1H), 2.86 (dd, $J = 8.8$ and 12.9 Hz, 1H), 4.82 (s, 1H, N-H _{Deutr. Exch.}), 5.09 (dd, $J = 8.9$ and 3.8 Hz, 1H), 5.42 (s, 2H, CH ₂), 7.68–7.78 (m, 12H, Ar-H & Thioph. _(C3,4) -2H), 8.18 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	40.1, 76.1 (2CH ₂), 52.2 (CH), 113.4, 120.8, 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 129.5, 131.1, 140.6 (C-Ar), 142.6, 144.6, 164.5 (3 C=N-), 160.2 (C=O), 178.9 (C=S).
15b	686 (C-S-C), 1610-1625 (3 C=N), 1646 (amidic C=O), 1285 C=S), 3221 (N-H).	2.43 (s, 3H, Me), 2.82 (dd, $J = 13.2$ and 4.4 Hz, 1H), 2.87 (dd, $J = 8.9$ and 12.8 Hz, 1H), 4.81 (s, 1H, N-H _{Deutr. Exch.}), 5.10 (dd, $J = 8.7$ and 3.6 Hz, 1H), 5.41 (s, 1H, CH ₂), 7.65–7.79 (m, 11H, Ar-H & Thioph. _(C3,4) -2H), 8.20 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	
15c	686 (C-S-C), 1610-1625 (3 C=N), 1646 (amidic C=O), 1285 C=S), 3221 (N-H).	2.33 (s, 3H, Me), 2.81 (dd, $J = 13.2$ and 4.4 Hz, 1H), 2.89 (dd, $J = 8.9$ and 12.8 Hz, 1H), 4.80 (s, 1H, N-H _{Deutr. Exch.}), 5.09 (dd, $J = 8.7$ and 3.6 Hz, 1H), 5.38 (s, 1H, CH ₂), 7.65–7.79 (m, 11H, Ar-H & Thioph. _(C3,4) -2H), 8.22 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	

Table 2 continued

Comp.	IR (KBr) ν max in cm^{-1}	^1H -NMR (DMSO- d_6) in ppm	^{13}C -NMR (DMSO- d_6) in ppm
15d	685 (C-S-C), 1610-1620 (3 C=N), 1643 (amidic C=O), 1291 C=S, 3281 (N-H).	2.83 (dd, $J = 12.8$ and 4.1 Hz, 1H), 2.90 (dd, $J = 8.8$ and 12.7 Hz, 1H), 3.81 (s, 3H, OMe), 4.81 (s, 1H, N-H _{Deutr. Exch}), 5.10 (dd, $J = 8.7$ and 3.6 Hz, 1H), 5.40 (s, 1H, CH ₂), 7.60–7.79 (m, 11H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.4$, 1H, Thioph. _(C5) -H).	
15e	680 (C-S-C), 1613-1624 (3 C=N), 1641 (amidic C=O), 1290 C=S, 3283 (N-H).		
15f	681 (C-S-C), 1610-1620 (3 C=N), 1643 (amidic C=O), 1291 C=S, 3163 (N-H).	2.84 (dd, $J = 12.8$ and 4.1 Hz, 1H), 2.94 (dd, $J = 8.8$ and 12.7 Hz, 1H), 4.83 (s, 1H, N-H _{Deutr. Exch}), 4.09 (dd, $J = 8.7$ and 3.6 Hz, 1H), 5.43 (s, 1H, CH ₂), 7.10–8.09 (m, 11H, Ar-H & Thioph. _(C3,4) -2H), 8.20 (d, $J = 4.4$, 1H, Thioph. _(C5) -H).	
15g	715 (C-Cl), 681 (C-S-C), 1610-1620 (3 C=N), 1643 (amidic C=O), 1291 C=S, 3163 (N-H).		
15h	681 (C-S-C), 1610-1620 (3 C=N), 1390, 1520 (NO ₂), 1643 (amidic C=O), 1291 C=S, 3253 (N-H).	2.83 (dd, $J = 12.8$ and 4.1 Hz, 1H), 2.90 (dd, $J = 8.8$ and 12.7 Hz, 1H), 4.81 (s, 1H, N-H _{Deutr. Exch}), 5.10 (dd, $J = 8.7$ and 3.6 Hz, 1H), 5.45 (s, 1H, CH ₂), 7.60–8.09 (m, 11H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.4$, 1H, Thioph. _(C5) -H).	
15i	681 (C-S-C), 1610-1620 (3 C=N), 1390, 1520 (NO ₂), 1643 (amidic C=O), 1291 C=S, 3253 (N-H).		

Table 2 continued

Comp.	IR (KBr) ν max in cm^{-1}	$^1\text{H-NMR}$ ($\text{DMSO-}d_6$) in ppm	$^{13}\text{C-NMR}$ ($\text{DMSO-}d_6$) in ppm
15j	681 (C-S-C), 1610-1620 (3 C=N), 1390, 1520 (NO_2), 1643 (amidic C=O), 1291 (C=S), 3253 (N-H).	2.83 (dd, $J = 12.8$ and 4.1 Hz, 1H), 2.90 (dd, $J = 8.8$ and 12.7 Hz, 1H), 3.81 (s, 3H, OMe), 4.81 (s, 1H, N-H Deutr. Exch), 5.10 (dd, $J = 8.7$ and 3.6 Hz, 1H), 5.40 (s, 1H, CH_2), 7.60–7.79 (m, 11H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.4$, 1H, Thioph. _(C5) -H).	40.1, 76.1 (2CH_2), 52.2 (CH), 113.4, 120.8, 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 129.5, 131.1, 140.6 (C-Ar), 142.6, 144.6, 164.5 (3 C=N-), 160.2 (C=O), 178.9 (C=S).
16	687 (C-S-C), 1610-1620 (4 C=N), 1686 (amidic C=O), 2218 (C $\equiv$ N).	2.81 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.90 (dd, $J = 9.2$ and 13.5 Hz, 1H), 3.83 (s, 2H, CH_2), 5.16 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	13.1, 40.1 (2CH_2), 52.5 (CH), 127.0, 127.6, 128.1, 128.2, 128.8, 129.3, 131.1, 140.5 (C-Ar), 153.5, 157.6, 160.5, 164.5 (4 C=N-), 160.3 (C=O).
17	685 (C-S-C), 1611- 1625 (4 C=N), 1645 (amidic C=O).	2.43 (s, 3H, Me), 2.81 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.89 (dd, $J = 9.2$ and 13.5 Hz, 1H), 5.14 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.25 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	
18	680 (C-S-C), 1614- 1630 (4 C=N), 1641 (amidic C=O).	2.45 (s, 3H, Me), 2.83 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.92 (dd, $J = 9.2$ and 13.5 Hz, 1H), 5.16 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 12H, Ar-H & Thioph. _(C3,4) -2H), 8.22 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	
19	685 (C-S-C), 1611- 1625 (4 C=N), 1331 (S=O), 1645 (amidic C=O).	2.80 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.90 (dd, $J = 9.2$ and 13.5 Hz, 1H), 5.11 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.65–7.79 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 10.01 (s, 1H, NH Deutr. Exch).	

Table 2 continued

Comp.	IR (KBr) ν max in cm^{-1}	$^1\text{H-NMR}$ ($\text{DMSO-}d_6$) in ppm	$^{13}\text{C-NMR}$ ($\text{DMSO-}d_6$) in ppm
20	688 (C-S-C), 715 (C-Cl), 1610-1626 (2 C=N), 1641-1682 (3 amidic C=O).	2.82 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.91 (dd, $J = 9.5$ and 13.8 Hz, 1H), 4.21 (s, 2H, CH_2), 5.10 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.04 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	41.0, 42.4 (2 CH_2), 51.8 (CH), 127.1, 127.4, 128.0, 128.2, 128.5, 129.4, 131.1, 140.5 (C-Ar), 144.6, 164.5 (2 C=N-), 157.1, 159.0, 165.7 (3C=O).
21	686 (C-S-C), 1610-1626 (2 C=N), 1641-1682 (3 amidic C=O), 3186- 3350 (NH, NH_2).	2.79 (dd, $J = 13.7$ and 4.7 Hz, 1H), 2.86 (dd, $J = 9.5$ and 13.8 Hz, 1H), 3.12 (s, 2H, CH_2), 4.50 (s, 2H, $\text{NH}_{2\text{Deutr. Exch}}$), 5.10 (dd, $J = 9.5$ and 3.9 Hz, 1H), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H, 8.04 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 8.31 (s, 1H, $\text{NH}_{\text{Deutr. Exch}}$).	
22	685 (C-S-C), 1610-1620 (3 C=N), 1665-1686 (2amidic C=O), 2218 (C $\equiv$ N), 3186- 3350 (NH, NH_2).	2.83 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.94 (dd, $J = 9.2$ and 13.5 Hz, 1H), 3.34 (s, 2H, Pyridaz. _(C6) -2H), 5.12 (dd, $J = 9.5$ and 3.9 Hz, 1H), 6.81 (s, H, $\text{NH}_{\text{Deutr. Exch}}$), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 8.50 (s, 2H, $\text{NH}_{2\text{Deutr. Exch}}$).	
23	685 (C-S-C), 1615-1630 (4 C=N), 1662, 1679 (2amidic C=O), 3251 (NH), 3453 (br OH).	1.04 (s, 3H, Me), 2.79 (dd, $J = 13.5$ and 4.5 Hz, 1H), 2.87 (dd, $J = 9.2$ and 13.5 Hz, 1H), 3.32 (s, 2H, Pyridaz. _(C6) -2H), 5.10 (dd, $J = 9.5$ and 3.9 Hz, 1H), 6.92 (s, H, $\text{NH}_{\text{Deutr. Exch}}$), 7.67–7.78 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 9.31 (s, 1H, $\text{OH}_{\text{Deutr. Exch}}$).	
27	679, 685 (2C-S-C), 1610-1620 (3 C=N), 1665-1686 (2amidic C=O), 3215 (NH $_2$).	2.78 (dd, $J = 12.4$ and 4.6 Hz, 1H), 2.91 (dd, $J = 8.1$ and 12.1 Hz, 1H), 5.01 (dd, $J = 8.6$ and 3.8 Hz, 1H), 6.21 (s, 1H, Thiaz. _(C4) -H), 6.51 (s, 2H, $\text{NH}_{2\text{Deutr. Exch}}$), 7.68–7.79 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H).	

Table 2 continued

Comp.	IR (KBr) V max in cm^{-1}	$^1\text{H-NMR}$ ($\text{DMSO-}d_6$) in ppm	$^{13}\text{C-NMR}$ ($\text{DMSO-}d_6$) in ppm
30	679, 689 (2C-S-C), 1610-1620 (C=N), 1651-1686 (3 amidic C=O), 3185 (NH).	2.78 (dd, $J = 12.4$ and 4.6 Hz, 1H), 2.92 (dd, $J = 8.4$ and 12.3 Hz, 1H), 5.01 (dd, $J = 8.6$ and 3.8 Hz, 1H), 6.83 (s, 1H, Thiaz. _(C5) -H), 7.68–7.79 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 10.13 (s, 1H, $\text{NH}_{\text{Deutr. Exch}}$).	
32	685 (C-S-C), 1610-1620 (4 C=N), 1672 (amidic C=O), 2219 (C≡N), 3186-3325 (NH, NH_2).		
33	681, 690 (2C-S-C), 1610-1620 (4C=N), 1653, 1710 (2 C=O), 3185-3215 (2 NH).	2.51 (s, 3H, Me), 2.72 (s, 3H, MeCO), 2.82 (dd, $J = 12.4$ and 4.6 Hz, 1H), 2.93 (dd, $J = 8.4$ and 12.3 Hz, 1H), 5.01 (dd, $J = 8.6$ and 3.8 Hz, 1H), 7.68–7.79 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 10.21, 10.83 (s, 2H, $2\text{NH}_{\text{Deutr. Exch}}$).	18.4, 25.7 (2Me), 41.0 (CH_2), 52.3 (CH), 127.1, 127.4, 128.0, 128.2, 128.5, 129.4, 131.1, 140.5 (C-Ar), 144.6, 158.0, 158.3, 164.5 (4 C=N-), 160.3, 192.6 (C=O).
35	681, 694 (2C-S-C), 1610-1620 (4C=N), 1653, 1710, 1730 (3 C=O), 3185-3215 (2 NH).	2.72 (s, 3H, MeCO), 2.82 (dd, $J = 12.4$ and 4.6 Hz, 1H), 2.93 (dd, $J = 8.4$ and 12.3 Hz, 1H), 3.77 (s, 3H, Me), 5.01 (dd, $J = 8.6$ and 3.8 Hz, 1H), 6.20 (s, 1H, =C-H), 7.68–7.79 (m, 7H, Ar-H & Thioph. _(C3,4) -2H), 8.21 (d, $J = 4.5$, 1H, Thioph. _(C5) -H), 10.21, 10.81 (s, 2H, $2\text{NH}_{\text{Deutr. Exch}}$).	

2.2 Pharmacological evaluation

2.2.1 Anti-microbial impact

Selected twelve newly synthesized compounds (**15a-j**, **7** and **14**), were screened for their *in vitro* antimicrobial activity, against diverse bacterial and fungal strains; two Gram-positive species namely *Bacillus subtilis* ATCC 6633 (BS) and *Staphylococcus aureus* ATCC 6535 (SA), and two Gram-negative species, *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Escherichia coli* ATCC 8739 (EC). Also, they were screened against two fungal strains namely, *Candida albicans* ATCC 10231 (CA), and *Aspergillus brasiliensis* ATCC 16404 (AB). The reference drug for the evaluation of antibacterial activity against Gram positive bacteria was *Ampicillin*, and *Gentamicin* was the reference in assessing the activity against Gram negative bacteria, while *Amphotericin B* was taken as the stander for the anti-fungal impact. The inhibitory effects of the synthesized compounds versus these organisms, are depicted in **Table 3**. The parent precursors **7** and **14**, were the lowest potent anti the evaluated species, as compared to the title compounds **15a-j**. The anti-microbial data presented in **Table 3** declared that, the majority of the evaluated compounds exhibited proficient inhibitory effects on the growth of the tested microorganisms. In general, most of the screened compounds revealed better potency contra Gram-positive bacteria than Gram-negative ones [39, 40]. This result is supported by the fact that, Gram-positive bacteria have a thick cell wall consisting multilayered peptidoglycan, while, in Gram-negative bacteria the cell wall is very thin, consisting of very few layers of peptidoglycan along with a second layer of lipopolysaccharides and lipoproteins. This outer membrane is selectively permeable and thus, control access to the underlying structures. This renders the Gram-negative bacteria generally less susceptible to tested compounds than the Gram-positive bacteria. In addition, the outcome of the anti-microbial investigation declared that, majority of the tested compounds showed less potency against fungal species comparable to bacterial species. As evident from **Table 3**, compounds (**15j**, **15f**, **15i**, **15h** and **15g**), showed the proficient potency with MIC values ≤ 14.15 and $17.13 \mu\text{g/ mL}$ in hindering the growth of (SA) and (BS) respectively. Compound **15j** was the most powerful with MIC values 11.32 and $11.34 \mu\text{g/ mL}$, to be nearly as effective as the positive control *Ampicillin* in hindering the growth of both (SA) and (BS), respectively. Compounds (**15a**, **15b** and **15c**) showed moderate activity with MIC values ($24.25 \mu\text{g/ mL}$) in inhibiting the growth of the two Gram-negative strains (SA and BS). While, compounds (**15d**, **7** and **14**), showed the lowest potency among the tested compounds with MIC values (30.82 - $39.93 \mu\text{g/mL}$) against Gram-negative bacteria.

On the other hand, for the antifungal potency of the tested compounds the trend alters (**Table 3**), compounds (**15d**, **15c**, **15e** and **15b**), showed the highest antifungal activity with MIC values (14.32 - $17.25 \mu\text{g/mL}$), comparable to *Amphotericin B*. Compounds **15d**, emerged as the most powerful

antifungal analogue with MIC values 14.32 and 14.15 $\mu\text{g/mL}$ in hindering the growth of (**AB**) and (**CA**) respectively. While, compounds (**15j**, **15f**, **15i** and **15h**), exhibited moderate antifungal activity, against the tested organisms with MIC values (18.36-24.15 $\mu\text{g/mL}$). Compounds (**15g**, **15a**, **14** and **7**), showed the lowest antifungal activity against the tested organisms comparable to *Amphotericin B* with MIC values $> 30.05 \mu\text{g/mL}$.

Table 3: Antimicrobial evaluation of some synthesized compounds using the disc-diffusion method.

Comp. No. (200 $\mu\text{g/mL}$)	MIC values, $\mu\text{g/mL}^a$ (Inhibition zone (mm))					
	Gram positive bacteria		Gram negative bacteria		Fungal	
	SA	BS	PA	EC	AB	CA
7	36.25 (25)	37.55 (25)	39.93 (25)	38.25 (25)	45.35 (20)	43.21 (23)
14	32.45 (24)	32.91 (24)	35.01 (24)	34.72 (25)	37.85 (23)	36.80 (23)
15a	16.82 (30)	17.01 (29)	18.16 (29)	17.82 (30)	33.24 (24)	33.15 (25)
15b	19.00 (29)	19.52 (29)	20.62 (28)	20.15 (29)	17.25 (30)	16.17 (30)
15c	22.62 (28)	22.85 (28)	24.25 (29)	23.31 (30)	14.65 (32)	14.58 (33)
15d	30.82 (26)	31.11 (25)	33.85 (26)	32.11 (26)	14.32 (33)	14.15 (33)
15e	27.06 (27)	28.15 (26)	30.01 (28)	29.32 (27)	15.16 (31)	15.08 (32)
15f	11.82 (34)	11.87 (33)	12.71 (33)	12.52 (32)	19.05 (28)	18.85 (29)
15g	14.15 (31)	14.31 (31)	17.13 (30)	16.23 (31)	30.21 (24)	30.05 (25)
15h	13.04 (32)	13.32 (31)	14.54 (31)	14.02 (32)	24.15 (25)	24.06 (26)
15i	12.10 (33)	12.16 (32)	13.35 (32)	13.23 (32)	21.02 (27)	20.10 (28)
15j	11.32 (34)	11.34 (34)	12.21 (33)	12.05 (33)	18.36 (29)	18.29 (28)
Control*	11.25 (35)	11.26 (34)	12.45 (33)	12.45 (35)	14.13 (33)	13.42(34)
DMSO	-	-	-	-	-	-

^a Values are the average of three readings.

* Ampicillin in the case of Gram-positive bacteria, Gentamicin in the case of Gram-negative bacteria, and Amphotericin B in the case of fungi.

Structure activity relationship (SAR)

Therefore, structure activity relationship (SAR) studies were discussed to determine the effect of substituents on antimicrobial activities of the newly synthesized compounds. The obtained results declared implicitly some definite and interesting facts about the structure activity relationship (SAR), of synthesized compounds. The substitution pattern of 1,2,4-triazole analogue is affected by the electronic environment, and hence anti-microbial potency was also altered. Thus, antimicrobial activity was substantially influenced by substitution pattern on the phenyl ring. Compounds (**15j**, **15f**, **15i**, **15h**

and **15g**), displayed significant anti-bacterial activity against the tested organisms, indicating that conjugates bearing electron-withdrawing (EWG) groups such as NO₂, F and Cl exhibited higher potency than the rest of other compounds. While, electron donating (EDG) substituents, enhanced the antifungal potency.

It worth mentioned that, the highest antibacterial activity was observed when the electron attracting substituents were at *para* position (**15j** and **15f**), rather than if they at *ortho* or *meta* positions (**15i**, **15h** and **15g**) in the phenyl ring. On the other hand, considering the antifungal activity, the trend altered and compound **15d** was the most potent antifungal analogy, having (MeO) group as an electron releasing substituent at *para* position in its phenyl ring.

3. Materials and methods

3.1. General Information

Reagents were purchased from Sigma Aldrich (Bayouni Trading Co. Ltd., Al-Khobar, Saudi Arabia) and used without further purification. Reaction progress was monitored by TLC on silica gel pre-coated F254Merck plates. Spots were visualized by ultraviolet irradiation. Melting points were determined on a Gallenkamp electro thermal melting point apparatus and are uncorrected. IR spectra were recorded as potassium bromide disks using Bruker-Vector 22 FTIR Spectrophotometer. The NMR spectra were recorded with a Varian Mercury VXR-300 NMR spectrometer at 300 and 75 MHz for ^1H and ^{13}C NMR spectra, respectively, using $\text{DMSO}-d_6$ as solvents. Mass spectra were recorded on a Hewlett Packard MS-5988 spectrometer at 70 eV. Elemental analyses were carried out at the Micro-analytical Center of Taif University, Taif, KSA.

2-Hydrazinyl-6-phenyl-4-(thiophen-2-yl)-4,5-dihydropyrimidine (**1**) was synthesized according to the reported work [35,36]. Physical, analytical and spectral data are given in tables 1 and 2.

3.2. Synthesis

8-Phenyl-6-(thiophen-2-yl)-6,7-dihydro-2H-pyrimido[2,1-c][1,2,4]triazine-3,4-dione (2). A mixture of compound **1** (0.27 g, 1 mmol) and oxalyl chloride (0.12 g, 1 mmol), in DMF (15 mL) containing Et_3N (0.5 mL), was heated under reflux for 8 h. After cooling, the reaction mixture was poured onto ice/ H_2O . The formed solid was collected by filtration and recrystallized from EtOH to give compound **2** as yellow powder.

2-(4-Oxo-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2H-pyrimido[2,1-c][1,2,4]triazin-3(4H)-ylidene)hydrazinecarbothioamide (3). A mixture of compound **2** (0.32 g, 1 mmol) and thiosemicarbazide (0.09 g, 1 mmol) and HCl (0.5mL) in EtOH (30 mL) was heated under reflux for 8 h. The mixture was filtered on hot and the cooled filtrate was poured onto H_2O . The formed solid was filtered, washed with H_2O , dried well, and recrystallized from EtOH to afford **3** as yellowish-brown powder.

3-Amino-7-phenyl-9-(thiophen-2-yl)-8H-pyrimido[2,1-c][1,2,4]triazolo[3,4-f][1,2,4]triazin-11(9H)-one (5). A mixture of compound **7** (0.33 g, 1 mmol), and KSCN (0.48 g, 5 mmol) was heated at reflux for 7 h in glacial AcOH (25 mL), and the reaction mixture was allowed to cool to rt, then poured into H_2O . The solid formed was collected by filtration, dried and recrystallized from EtOH to afford **5**, as yellow crystals,

3-Chloro-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-4H-pyrimido[2,1-c][1,2,4]triazin-4-one (6) and **3-hydrazinyl-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-4H-pyrimido[2,1-c][1,2,4]triazin-4-one (7)**. They were synthesized, according to our reported work [35,36].

4,8-Diphenyl-10-(thiophen-2-yl)-9,10-dihydropyrimido[2,1-c][1,2,4]triazino[3,4-f][1,2,4]triazin-12(2H)-one (8). To a solution of **7** (0.33 g, 1 mmol) in absolute EtOH (25 mL), phenacyl bromide (0.19 g, 1 mmol) was added. The mixture was refluxed for 7 h (monitored by TLC), then left to cool. The precipitate formed was filtered off, washed with MeOH and recrystallized from EtOH to afford **8** as yellow crystals.

2-(Hydroxymethyl)-4,8-diphenyl-10-(thiophen-2-yl)-9,10-dihydropyrimido[2,1-c][1,2,4]triazino[3,4-f][1,2,4]triazin-12(2H)-one (9). A mixture of **8** (0.86 g, 2 mmol) and CH₂O solution (0.12 mL, 4 mmol) in EtOH (30 mL) was refluxed for 8 h. The precipitate obtained on cooling was filtered, washed several times with H₂O and recrystallized to yield **8** as yellow crystals.

7-Phenyl-9-(thiophen-2-yl)-8H-pyrimido[2,1-c][1,2,4]triazolo[3,4-f][1,2,4]triazin-11(9H)-one (10). *Method B*: A mixture of compound **7** (0.33 g, 1 mmol) and HC(OEt)₃ (15 mL) was refluxed for 6 h. The obtained solid after cooling was filtered, dried and recrystallized from DMF to yield compound **10** (60%), as brown powder.

Method B: A solution of **7** (0.33 g, 1 mmol) and DMF-DMA (0.22 mL, 2 mmol) in dry xylene (25 mL) was heated under reflux for 8 h. The solvent was then removed in *vacuo* and the solid so obtained was triturated with petroleum ether, collected by filtration and recrystallized from EtOH to yield **10** (60%).

7-Phenyl-9-(thiophen-2-yl)-8,9-dihydro-2H-pyrimido[2,1-c][1,2,4]triazolo[3,4-f][1,2,4]triazine-3,11-dione (11). To an ice-cooled suspension of KOH (0.11 g, 2 mmol) in absolute EtOH (20 mL), compound **7** (0.66 g, 2 mmol) and ethyl chloroformate (2 mL) were added. The mixture was refluxed for 5 h, then allowed to cool and poured into H₂O. The precipitate that obtained was filtered off and recrystallized from MeOH to afford **11** as brown crystals.

3-(Chloromethyl)-7-phenyl-9-(thiophen-2-yl)-8H-pyrimido[2,1-c][1,2,4]triazolo[3,4-f][1,2,4]triazin-11(9H)-one (12). A mixture of **7** (0.33 g, 1 mmol) and chloroacetyl chloride (0.11 mL, 1 mmol) in dry dioxane (25 mL) in the presence of anhydrous K₂CO₃ (0.13 g, 1 mmol) was refluxed on a water bath for 8 h. The reaction mixture was evaporated under reduced pressure and the residue

was triturated with H₂O. The formed solid was filtered off, washed with H₂O, dried and recrystallized from EtOH to afford **12** as brown powder.

3-Mercapto-7-phenyl-9-(thiophen-2-yl)-8H-pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4]triazin-11(9*H*)-one (14). To a stirred suspension of compound **7** (0.66 g, 2 mmol) in EtOH (20 mL), ethanolic potassium hydroxide (20 mL, 0.01 mol) and CS₂ (5 mL) were added dropwise. The reaction mixture was then heated under reflux for 7 h. After cooling and evaporation of the solvent under reduced pressure, the potassium salt obtained was dissolved in H₂O and acidified with 2 N aqueous HCl. The precipitate obtained was filtered and recrystallized from EtOH to yield **14** as colorless powder.

2-(((Substitutedphenyl)amino)methyl)-7-phenyl-9-(thiophen-2-yl)-3-thioxo-8,9-dihydro-2H-pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4]triazin-11(3*H*)-ones (15a-j). General procedure: A mixture of the thiol compound **14** (0.38 g, 1 mmol), 37% HCHO solution (2 mL) and the selected substituted anilines (1 mmol) in EtOH (30 mL) containing few drops of HCl, was stirred at rt, for 7-10 h (monitored by TLC). The solid that precipitated was filtered off, washed with H₂O, dried and finally recrystallized from proper solvent to furnish the respective products (**15a-j**), physical, analytical and spectral data are given in tables **1** and **2**.

2-(11-Oxo-7-phenyl-9-(thiophen-2-yl)-9,11-dihydro-8H-pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4] triazin-3-yl)acetonitrile (16). A mixture of **7** (0.33 g, 1 mmol) and ethyl 2-cyanoacetate (0.11 mL, 1 mmol in EtOH (30 mL) containing piperidine (0.3 mL) was refluxed for 8 h then left to cool at rt. The solid product was filtered off, washed with cold EtOH and recrystallized from EtOH to afford **16** as pale-yellow crystals.

3-Methyl-7-phenyl-9-(thiophen-2-yl)-8H-pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4]triazin-11(9*H*)-one (17). Compound **7** (0.33 g, 1 mmol), was dissolved in Ac₂O (15 mL) and stirred under reflux for 6 h, the reaction mixture was allowed to cool to rt and poured onto H₂O (50 mL). The resulted solid was filtered off, washed with H₂O several times, dried, and recrystallized using CHCl₃ to give **17** as brown powder.

3,7-Diphenyl-9-(thiophen-2-yl)-8H-pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4]triazin-11(9*H*)-one (18). A mixture of compound **7** (0.33 g, 1 mmol), and benzoyl chloride (15 mL) was heated at reflux for 4 h, the reaction mixture was allowed to cool to rt, the resulted solid was filtered off, washed with cold MeOH, dried, and recrystallized using EtOH to yield **18** as orange crystals.

7-Phenyl-9-(thiophen-2-yl)-8,9-dihydropyrimido[2,1-*c*][1,2,3,5]thiatriazolo[4,5-*f*][1,2,4]triazin-11(2*H*)-one 3-oxide (19). A solution of compound **7** (0.33 g, 1 mmol) in EtOH (25 mL), was treated with EtONa (0.23 g, 10 mmol sodium metal in 5 mL absolute EtOH), and thionyl chloride was heated on water bath for 8 h. The mixture was diluted with H₂O and extracted with ethyl acetate, the organic layers were dried over anhydrous Na₂SO₄ and the ethyl acetate was removed under reduced pressure, and recrystallized by EtOH to furnish **19** as yellow solid.

2-(2-Chloroacetyl)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (20). A mixture of chloroacetyl chloride (0.02 mol) in absolute EtOH (20 mL), was added dropwise during 30 min., to a well stirred solution of **2** (0.32 g, 1 mmol) in absolute EtOH (30 mL), containing Et₃N (0.5 mL). The mixture was stirred for further 1 h, at 80 °C, the reaction mixture was allowed to cool to rt, then poured onto ice-cooled H₂O. The produced solid was filtered off, dried and recrystallized from EtOH/dioxane (2:1) to afford **20** as yellowish-green powder.

2-(2-Hydrazinylacetyl)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (21). It was prepared according to the general procedure described for compound **7** as reddish-brown powder from EtOH in 76% yield.

3-Amino-5-(3,4-dioxo-8-phenyl-6-(thiophen-2-yl)-3,4,6,7-tetrahydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazin-2-yl)-1,6-dihydropyridazine-4-carbonitrile (22). To a solution of **21** (0.78 g, 1 mmol) in pyridine (15 mL), malononitrile (0.12 g, 2 mmol) was added, the mixture was heated under reflux for 6 h. the reaction mixture was left to cool at rt. The precipitate that formed was collected and recrystallized from CHCl₃ to yield **22** as yellow-orange powder.

2-(5-Hydroxy-7-methyl-2,3-dihydropyrimido[4,5-*c*]pyridazin-4-yl)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (23). A solution of **22** (0.44 g, 1 mmol) in Ac₂O/pyridine mixture (30 mL, (2:1)) was heated on a water bath for 8 h, then cooled and poured into ice/H₂O mixture. The precipitate thus obtained was filtered off, washed several times with H₂O, dried and recrystallized from EtOH to yield **23** as yellowish crystals.

2-(2-Aminothiazol-5-yl)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (27). A mixture of **20** (0.40 g, 1 mmol) and equimolar amount (1 mmol) of thiourea in dry EtOH (30 mL) containing anhydrous K₂CO₃ (0.13 g, 1 mmol) was refluxed for 8 h. The excess of solvent was removed under reduced pressure and the residue was triturated with MeOH. The

formed product was filtered, washed with cold MeOH, dried and recrystallized from the DMF-MeOH (1:3) to yield **27** as brown solid.

2-(2-Oxo-2,3-dihydrothiazol-4-yl)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2H-pyrimido[2,1-c][1,2,4]triazine-3,4-dione (30). A mixture of **20** (0.40 g, 1 mmol) and NH₄SCN (0.15 g, 2 mmol) in absolute EtOH (30 mL) was refluxed for 8 h. The reaction mixture was allowed to stand overnight, the solid produced was filtered, then recrystallized from EtOH to furnish **30** as yellow crystals.

4-Amino-2-((4-oxo-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2H-pyrimido[2,1-c][1,2,4]triazin-3(4H)-ylidene)hydrazono)-6-phenyl-2H-1,3-thiazine-5-carbonitrile (32). A mixture of **3** (0.39 g, 1 mmol) and benzylidinemalononitrile (0.15 g, 1 mmol) in dioxane (20 mL) containing few drops of piperidine (0.3 mL) was stirred under reflux for 6 h. Then, the reaction mixture was left to cool to rt. The solid produced was collected, washed with MeOH, and recrystallized from DMF to give **32** as yellowish-green solid.

3-((5-Acetyl-4-methylthiazol-2(3H)-ylidene)hydrazono)-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2H-pyrimido[2,1-c][1,2,4]triazin-4(3H)-one (33). To a solution of compound **3** (0.39 g, 1 mmol) in EtOH (30 mL), 3-chloro-2,4-pentanedione (0.13 mL, 1 mmol) was added, the mixture was heated under reflux for 6 h. The reaction mixture was cooled down, diluted with H₂O (50 mL), and sodium acetate (0.49 g, 6 mmol) was added, and the mixture was stirred for 15 min at rt. The obtained product was filtered, washed with H₂O, dried and recrystallized from EtOH to afford **33** as yellow powder.

Methyl 2-(4-oxo-2-((4-oxo-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2H-pyrimido[2,1-c][1,2,4]triazin-3(4H)-ylidene)hydrazono)thiazolidin-5-ylidene)acetate (35). To a well stirred solution of **3** (0.39 g, 1 mmol) in dry dioxane (20 mL), a solution of dimethyl acetylenedicarboxylate (0.11 mL, 1 mmol) in dioxane (20 mL) was added dropwise. The mixture was stirred for further 2 h at rt., then the reaction mixture was heated at reflux for further, 8 h (the reaction was monitored by TLC analyses). The solvent was evaporated under *vacuum*, the formed solid was filtered off and then recrystallized from MeOH-H₂O to afford **35** as yellowish-brown powder.

3.3. Antimicrobial assessment

3.3.1 Methodology

The antimicrobial activity of the new synthesized compounds was evaluated using the disk-diffusion method [41]. A plate of 90 mm diameter containing the Muller-Hinton agar for the growth of bacteria and the sabouraud dextrose agar, for the growth of fungi were prepared and each plate was

separately inoculated with different cultures, of the test bacteria and fungi by swabbing aseptically on the whole surface of the agar with cotton wool. A 6 mm diameter filter paper disc was saturated with 200 μg /mL of the tested compounds in DMSO. The discs were air dried and placed aseptically at the center of the plates. The plates were left in refrigerator for 1h, before incubation to allow the extract to diffuse into the agar. *Ampicillin* and *gentamicin* were used as bacterial standards, while, *amphotericin B* was used as fungal reference to evaluate the efficacy of the tested compounds, DMSO was used as negative control. After incubation of the plate at the suitable temperature (37 °C for bacteria and 25 °C for fungi), the results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the disks in mm. The minimum inhibitory concentration (MIC) measurement was determined for compounds exhibited significant growth inhibition zones more than 15 mm using two-fold serial dilution method [42]. The MIC (μg / mL) and IZ diameters values are recorded in **Table 3**.

4. Conclusions

In conclusion, a novel series of annelated pyrimido[2,1-*c*][1,2,4]triazolo[3,4-*f*][1,2,4]triazines, were synthesized and *in vitro* evaluated for their antimicrobial activity. Thus, 8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazine-3,4-dione (**2**), 2-(4-oxo-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-2*H*-pyrimido[2,1-*c*][1,2,4]triazin-3(4*H*)-ylidene)hydrazinecarbothioamide (**3**), and 3-hydrazinyl-8-phenyl-6-(thiophen-2-yl)-6,7-dihydro-4*H*-pyrimido[2,1-*c*][1,2,4]triazin-4-one (**7**), were synthesized as new two-pharmacophoric-motif precursors in considerable yields. The reactivity of the hydrazinyl tag and thioureido moieties were investigated in an assortment of manipulations involving cyclocondensation, aiming at annulation a new three-pharmacophoric-motif conjugates. The results of antimicrobial screening, declared that conjugates (**15j**, **15f**, **15i**, **15h**, **15g**, **15a**, **15b**, **15c**, **15e** and **15d**), in decreasing order exhibited significant antimicrobial activities, while, compounds (**7** and **14**), showed the less potency. Compounds with electron withdrawing moiety for instance NO₂ and fluoro at *para* and *meta* positions, exhibited the most significant antibacterial activity. It worth mentioning that, *para* position was more convenient for augmenting the antibacterial activity. Compounds **15d** with electron releasing group at *para* position, emerged as the most powerful antifungal analogue with MIC values 14.32 and 14.15 μg /mL in hindering the growth of (**AB**) and (**CA**), respectively.

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This work is dedicated to the spirit of my father (Helmy El Azab).

Author Contributions

Conceptualization: I.H.; performing the research: I.H and N.A.; writing, original draft preparation: N.A.; review, editing and supervision: I. H.

Conflicts of Interest

The authors declare that this article content has no conflict of interest.

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