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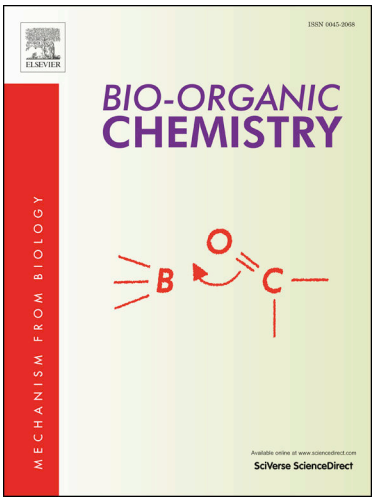

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Design, green one-pot synthesis and molecular docking study of novel *N*,*N*-bis(cyanoacetyl)hydrazines and bis-coumarins as effective inhibitors of DNA gyrase and topoisomerase IV

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Abstract: A novel, quick, environmentally safe, and one-pot synthesis of a series of *N*,*N*-bis(cyanoacetyl)hydrazine derivatives, bis-imino-2*H*-chromenes and bis-2-oxo-2*H*-chromene derivatives have been designed. Some selected newly synthesized compounds were investigated *in vitro* for their antibacterial activity. Compound **5j** is the most toxic compound against *Staphylococcus aureus* with activity index 171 %, followed by compound **15b** with activity index 136% compared to standard drug ampicillin. Moreover, compound **15a** is the most toxic compound against *Escherichia coli* with activity index 111% compared to standard drug gentamicin. Minimum inhibitory concentration (MIC) was carried out for compounds with high antibacterial activity. Compound **5j** has good MIC (7.8 μg/ml) against *Staphylococcus aureus* while **15a** has good MIC (31.25 μg/ml) against *Streptococcus mutans* which is better than MIC of the standard drug ampicillin (MIC = 62.5 μg/ml). Compounds **5j**, **5k**, **15a, 15b** and **15e** which have good MIC values were introduced to enzyme assay against DNA gyrase and topoisomerase IV. The results showed that compound **15a** can strongly inhibit DNA gyrase and topoisomerase IV (IC_{50} = 27.30 and 25.52 μM respectively), compared to methotrexate as the standard drug (IC_{50} = 29.01 and 23.55 μM respectively). Structure-activity relationships were also discussed based on the biological and docking simulation results.

Keywords: *N*,*N*-bis(cyanoacetyl)hydrazines; bis-coumarins; antibacterial; DNA gyrase and topoisomerase IV; molecular docking study; enzyme inhibition.

1. Introduction

Bactria resistance takes place in the presence of one or more antibiotics. When this occurs, the resistant bacteria continue to cause infection. Bacteria are able to resist antibiotics through many various ways [1-3]. The more diffusion of these resistant organisms happens, the more they add to the resistance genes in all bacteria; as a result, undefeatable strains of disease-causing microbes are generated. Therefore, the continuous designing and developing of new antibacterial drugs is necessary.

DNA gyrase and topoisomerase IV (Topo IV) are both IIA topoisomerases present in bacteria. They have similar structures and function as they exhibit 40% of overall DNA sequence identity [4] and possess significantly similar active sites [5]. Therefore, they are considered as attractive targets for designing new antibiotics [6]. They are highly homologous enzymes that play essential roles in bacterial DNA replication, chromosome segregation, and DNA compaction [7].

Both DNA gyrase and topoisomerase IV are heterotetramers consisting of two A and two B subunits (A2B2). The A subunit contains the active-site tyrosine involved in the DNA breakage-reunion activity while the B subunit catalyzes ATP hydrolysis [8]. Though clearly related, based on amino acid sequence similarity, they each play crucial, but distinct, roles in the cell.

DNA gyrase encoded by *GyrA* and *GyrB*; *GyrA* maps to 50.3 minutes on the *E. Coli* chromosome and encodes a 97 KDa protein [875amino acids] [9,10] whereas *GyrB* maps to 83.5 minutes and encodes a 89.9 KDa protein [804 amino acids] [9]. DNA gyrase has ability to introduce negative supercoils into DNA by looping the template so as to form a crossing, then cutting one of the double helices and passing the other through it before releasing the break, changing the linking number by two in each enzymatic step. This process occurs in prokaryotes, whose single circular DNA is cut by DNA gyrase and the two ends are then twisted around each other to form supercoils.

In spite of, topoisomerase IV binds to DNA as a heterotetramer consisting two *parC* and two *parE* genes [11]. Topoisomerase IV is a decatenating enzyme that resolves interlinked daughter chromosomes following DNA replication (Fig. 1) [12].

Topoisomerase inhibitors act as an obstacle to the ligation step in both gram positive and gram negative microbial cell cycle, generating single and double stranded breaks that damage the integrity of the genome leading to apoptosis (cell death) in the

proliferating cell [13]. Its absence in the mammalian organism and its crucial role in the bacterial DNA replication cycle make this enzyme a suitable target for the development of antibacterial drugs with selective toxicity.

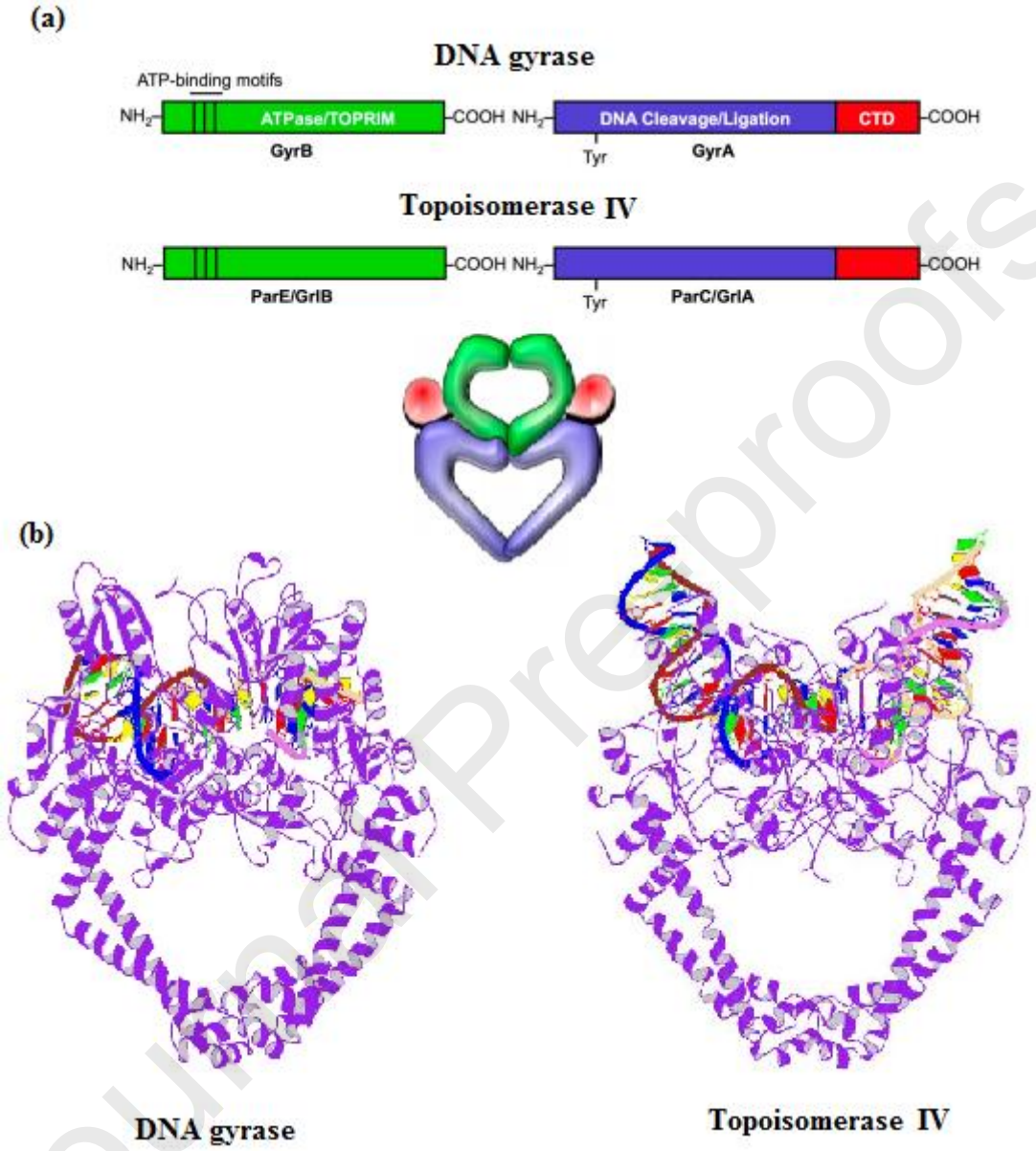


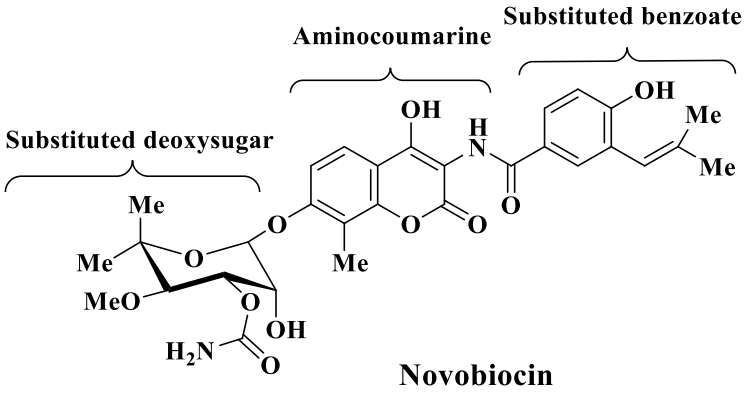
Fig. 1. Structure of type IIA topoisomerases (DNA gyrase and topoisomerase IV). (a) Primary domain structure of type IIA topoisomerases, (b) 3D Structure of DNA gyrase and topoisomerase IV with pdb entry [4Z2C for DNA gyrase] and [3FOE for topoisomerase IV].

The best characterized inhibitor classes of type IIA topoisomerases are the fluoroquinolone antibiotics which act by stabilizing the complex between a DNA molecule and the ParC/GyrA active site of the enzyme [14,15]. However, the

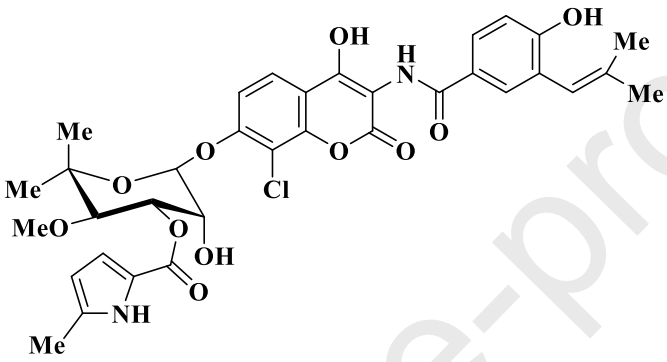
increasing prevalence of fluoroquinolone resistant strains and problems with side effects are beginning to limit the utility of this widely utilized antibiotic class [16].

On the other hand, aminocoumarins, catalytic inhibitors of DNA gyrase act *via* competitive inhibition of GyrB ATPase activity [17]. Aminocoumarin antibiotics, such as novobiocin, clorobiocin, and coumermycin A1 (Fig. 2) are effective at killing Gram-positive bacteria, but were unsuccessful in the clinic. A number of structure-based design efforts have been effective in generating ligand efficient inhibitors superior to aminocoumarin antibiotics in potency, but to date, no synthetic or natural product GyrB/ParE inhibitors have reached the clinic. Thus, the GyrB/ParE ATP-binding subunits represent unexploited targets for the development of novel antibiotics.

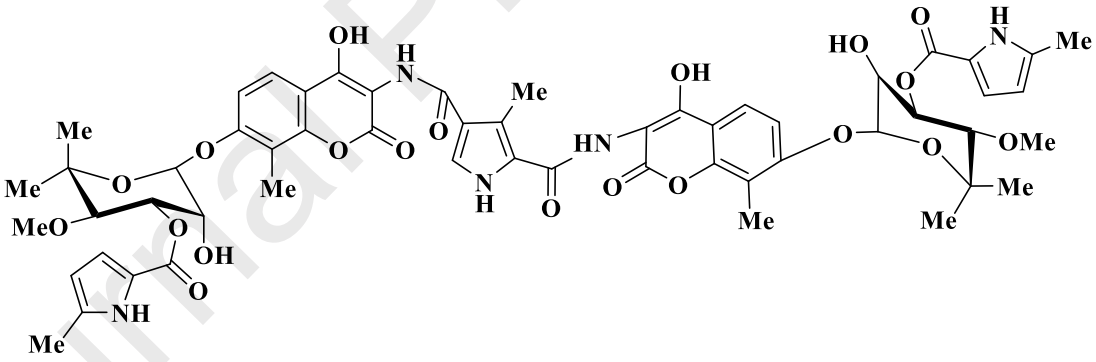
Derivatives of 2-cyanoacetohydrazide has been regarded as important intermediates and starting materials in the synthesis of many remarkable heterocyclic ring systems as they are highly reactive and polyfunctional compounds due to having both electrophilic and nucleophilic centers. Furthermore, they have received a great attention as pharmaceutical drugs [18, 19] and as antibacterial agents [20]. Moreover, they are applicable in the industrial field in the production of herbicides [21] and dyes [22]. 2-Cyanoacetohydrazide derivatives have been utilized in the synthesis of coumarins [23,24], quinolines [25], pyrazoles [26], pyrazolopyrimidines [27], thiazoles [28], pyridines [29], pyrimidines [30] and many other heterocyclic ring systems [31]. In view of these reports, and in continuation to our work in the synthesis of novel biologically active compounds [32-38], we designed a green synthesis of a series of some novel bis-coumarin analogs aiming to introduce new DNA gyrase and topo IV inhibitors as antibacterial agents starting from the key intermediate *N*,*N*-bis(cyanoacetyl)hydrazines.



Novobiocin



clorobiocin



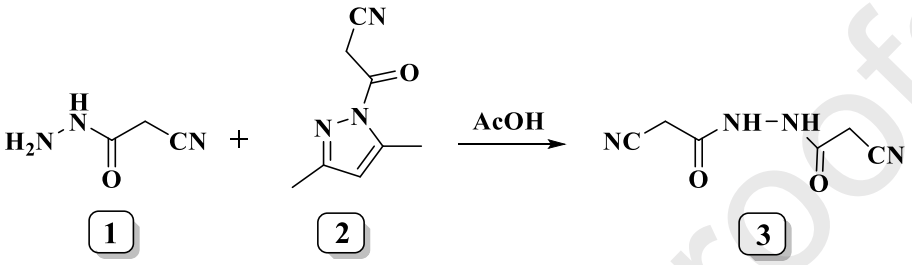
Coumermycin A1

Fig. 2. Aminocoumarin inhibitors

2. Results and discussion

2.1. Chemistry

The reaction of 2-cyanoacetohydrazide (**1**) with 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile (**2**) in glacial acetic acid leads to the formation of *N*,*N*-bis(cyano acetyl)hydrazine **3** (Scheme 1). The structural assignment of compound **3** was performed based on elemental analyses and spectral data (see experimental).



Scheme 1

Knoevenagel condensation of compound **3** with various aldehyde derivatives **4a-k** *via* grinding in the presence of sodium hydroxide as a basic catalyst at room temperature afforded the respective arylmethylene derivatives **5a-k** (Scheme 2). The structures of the desired products were confirmed by elemental analyses and spectral data (IR, ^{1}H NMR and MS). For example, the IR spectrum of **5b** showed an absorption bands at 3432, 2210 and 1649 cm^{-1} corresponding to NH, CN and CO group. The ^{1}H NMR spectrum of **5b** revealed two singlet signals at δ = 10.64 and 7.94 ppm attributable to NH and vinylic protons, respectively. Mass spectrum exhibited a molecular ion peak at *m/z* 402 (M^+) which is consistent with the molecular formula $C_{22}H_{18}N_4O_4$. Elemental analyses together with mass spectrum is in agreement to the proposed structures (Scheme 2).

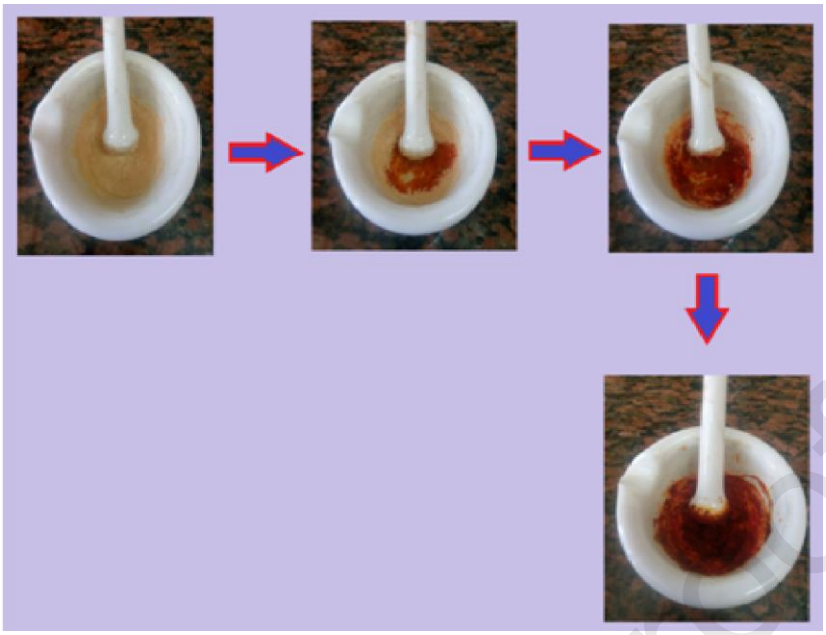
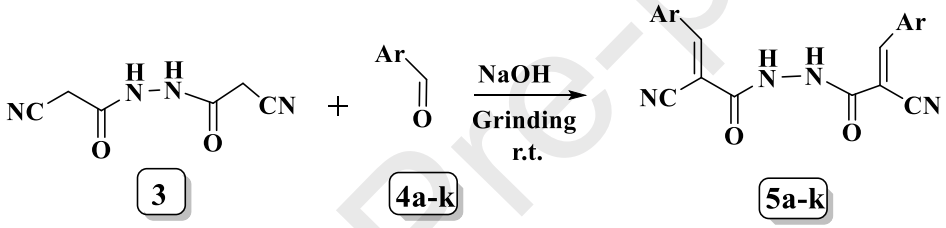


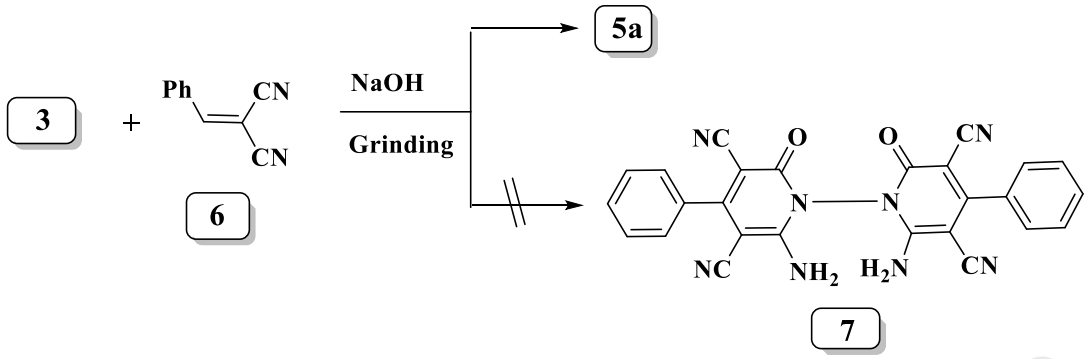
Fig. 3. Appearance of reaction media at different times for compound **5a**



4,5a, Ar = C_6H_5
b, Ar = 2-$OMeC_6H_4$
c, Ar = 4-ClC_6H_4
d, Ar = 2,5-$(OMe)_2C_6H_3$
e, Ar = 2,4-$(Cl)_2C_6H_3$
f, Ar = 3-OHC_6H_4
g, Ar = 2-FC_6H_4O
h, Ar = 2-thienyl
i, Ar = 2-furyl
j, Ar = 4-NO_2-2-furyl
k, Ar = 4-Me-2-furyl

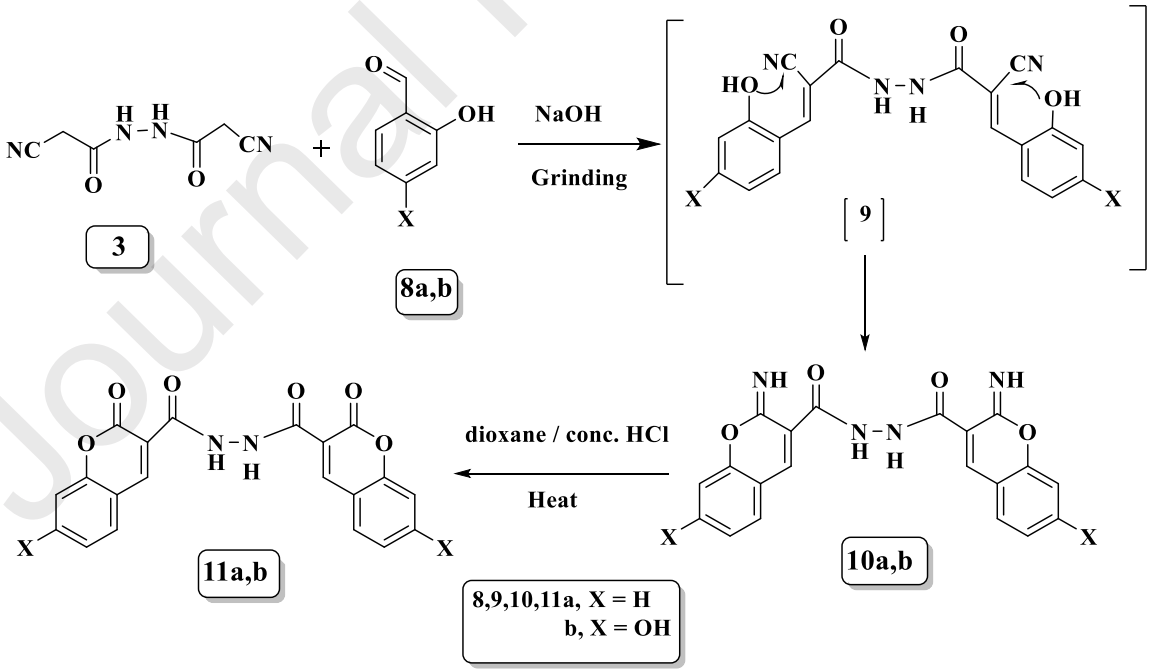
Scheme 2

Grinding compound **3** with 2-benzylidenemalononitrile (**6**) in the presence of sodium hydroxide at room temperature provided compound **5a** not compound **7** as proved by mp., m.mp. and spectral data (Scheme 3).



Scheme 3

When compound **3** was grand with *o*-hydroxybenzaldehydes **8a,b** in sodium hydroxide, products **10a,b** were isolated (Scheme 4). The IR spectrum of **10a** exhibited absorption band at 3433 cm^{-1} due to the NH function group and another absorption band at 1669 cm^{-1} corresponding to CO group. Its ^{1}H NMR spectrum presented D_2O exchangeable signals at 10.25 and 10.68 ppm owing to NH protons, along with the signals for aryl protons in expected region. The latter compound was refluxed in dioxane with few drops of concentrated hydrochloric acid to yield the respective bis-coumarin derivatives **11a,b**. The structures of the products were established on the basis of elemental analysis and spectral data (Scheme 4).



Scheme 4

Condensation of compound **3** with different azosalicylaldehydes **12a-e** under the same reaction conditions furnished the isolated products **14a-e**. Refluxing these products in dioxane and few drops of conc. HCl afforded products **15a-e** (Scheme 5). The structure of the isolated products was supported by elemental analysis and spectral data (IR, ^{1}H NMR and MS). For example, the IR spectrum of the isolated product **15e** showed absorption bands at 3432 and 1665 cm^{-1} due to NH and CO groups, respectively. The ^{1}H NMR spectrum of this product revealed a singlet signal at δ = 10.46 ppm attributable to NH, besides the aryl protons. Mass spectrum showed a molecular ion peaks at *m/z* 620 (M^{+}) which is consistent with the assigned structure **15e**.

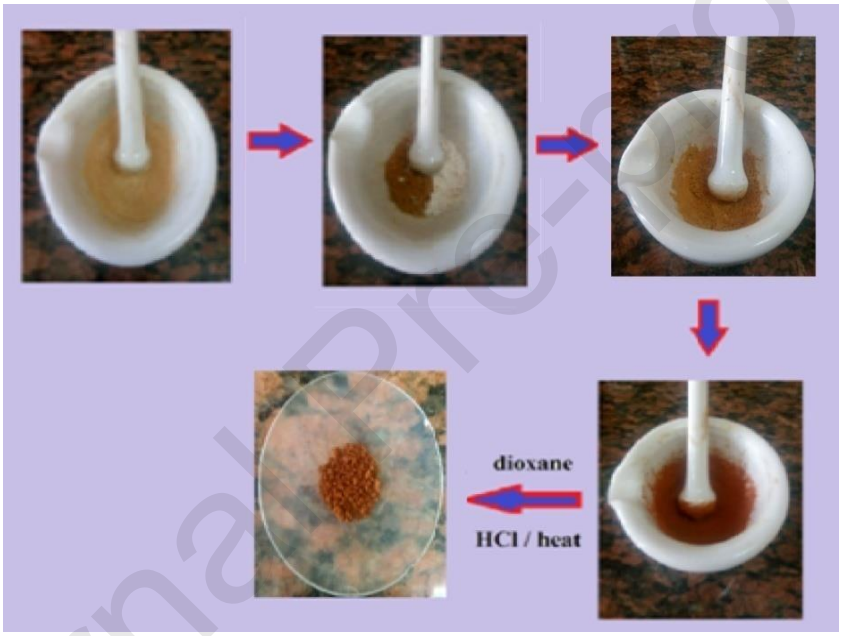
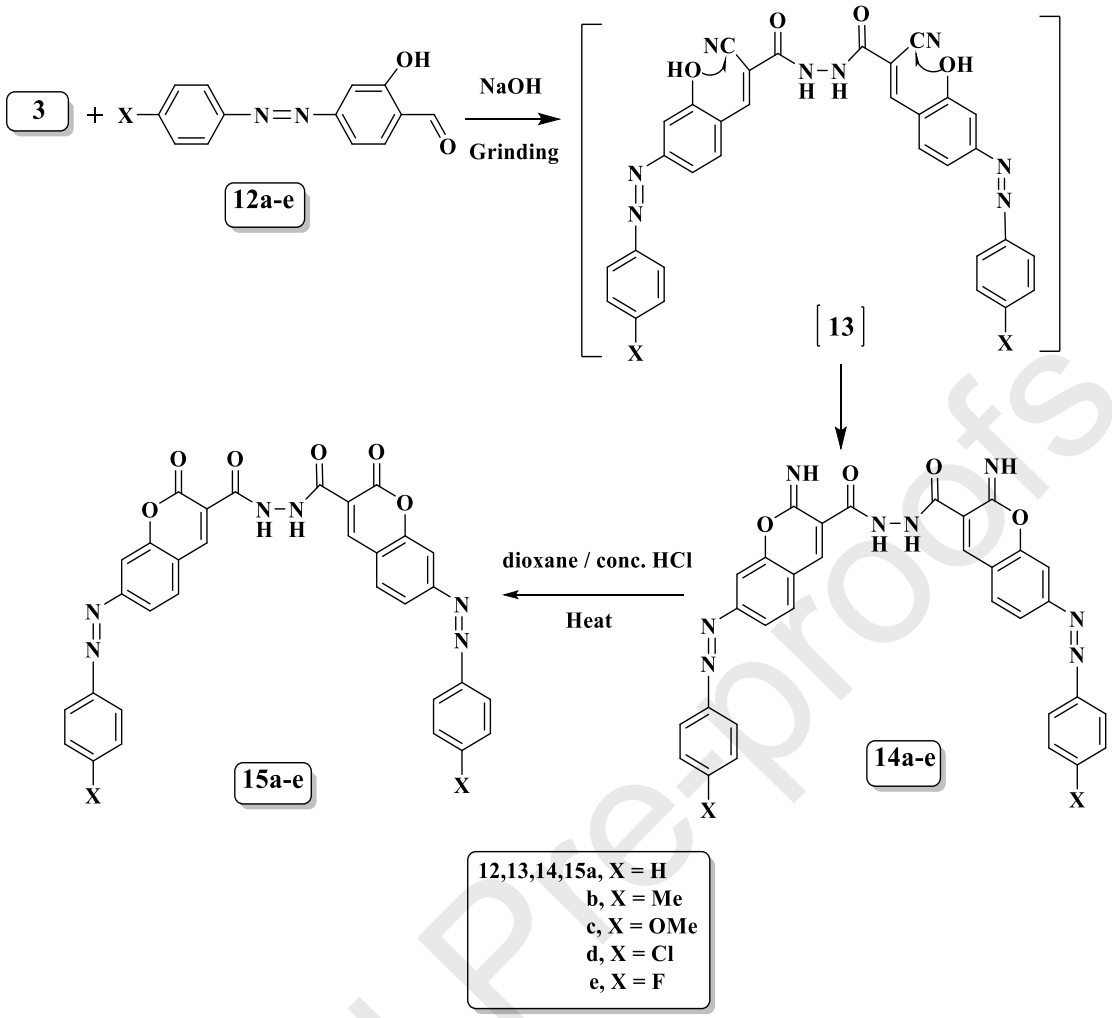
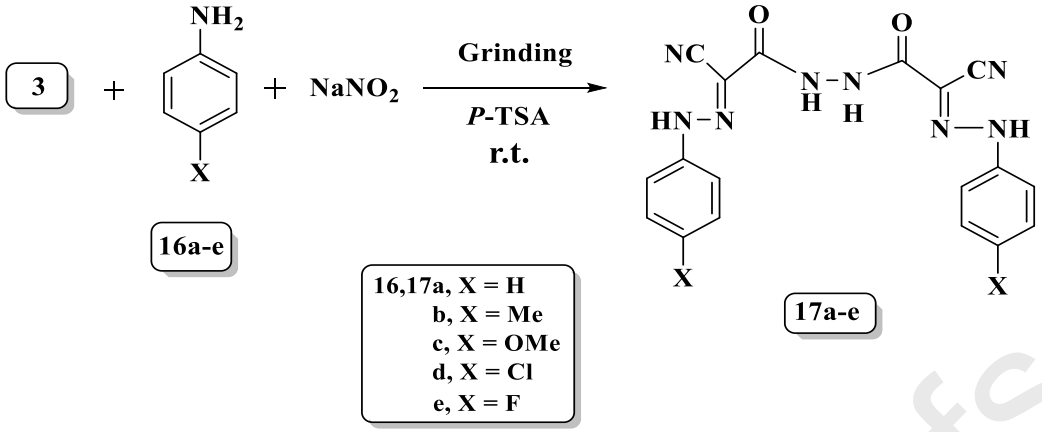


Fig. 4. Appearance of reaction media at different times for compound **15a**



Scheme 5

It is interesting to synthesize azo dyes by grind stone method. Thus, a mixture of equimolar amounts of compound **3**, aromatic amines **16a-e** and sodium nitrite in presence of solid *p*-toluenesulfonic acid (*P*-TSA) was grand to afford colored products **17a-e**, (Scheme 6). The structure of the products **17a-e** was proved by elemental analysis and spectral data (IR, ^{1}H NMR, ^{13}C NMR and MS). For example, the IR spectrum of the isolated product **17e** showed absorption bands at 3433, 3215, 2216 and 1670 cm^{-1} corresponding to NH, CN and CO groups, respectively. The ^{1}H NMR spectrum of the same product revealed two singlet signals at δ = 10.23 and 11.28 ppm due to NH protons beside, the aryl proton signals in the expected regions. Mass spectrum showed a molecular ion peaks at *m/z* 410 (M^{+}) which is consistent with the molecular formula $C_{18}H_{12}F_2N_8O_2$. The ^{13}C NMR spectrum of **17e** exhibited the following characteristic signals 105.7, 110.9, 115.1, 115.9, 128.0, 138.6, and 160.7 ppm.



Scheme 6

2.2. Biology

2.2.1. Antibacterial evaluation

Some selected newly synthesized compounds **3**, **5a**, **5b**, **5c**, **5d**, **5e**, **5h**, **5i**, **5j**, **5k**, **14a**, **14b**, **14c**, **14d**, **14e**, **15a**, **15b**, **15c**, **15d**, **15e**, **17c**, **17d** and **17e** were tested *in vitro* for their antibacterial activity against *Staphylococcus aureus* and *Streptococcus mutans* (Gram positive bacteria), *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Gram negative bacteria) using nutrient agar medium. Ampicillin and gentamicin were used as standard drugs for Gram positive and Gram negative, respectively. The antimicrobial activity of the newly synthesized compounds was determined using agar well diffusion method [39], and the diameters (mm) of inhibition zones are shown in Table 1.

From the results presented in Table 1, it was found that most of the tested compounds exhibited toxicity against *S. aureus*, *S. mutans* and *E. coli* higher than *P. aeruginosa* and *K. pneumonia*. Compound **5j** is the most toxic compound against *S. aureus* with activity index 171% followed by compound **15b** with activity index 136%. In addition, compounds **15a** and **15e** with activity index 128 and 120% respectively compared to standard drug ampicillin. Moreover, compounds **15a**, **15b** and **15e** exhibited toxicity against fungi (*C. albicans*) with activity index 104, 74 and 60%, respectively compared to standard drug nystatin. Compound **15a** is the most toxic against *E. coli* with activity index 111% compared to gentamicin activity. Also, toxicity of compound **5j** is better than **15b** against *E. coli*, *K. pneumonia* and *P. aeruginosa* with activity index 98, 94 and 57% respectively. The remaining tested compounds showed activity between moderate and high compared to standard drug.

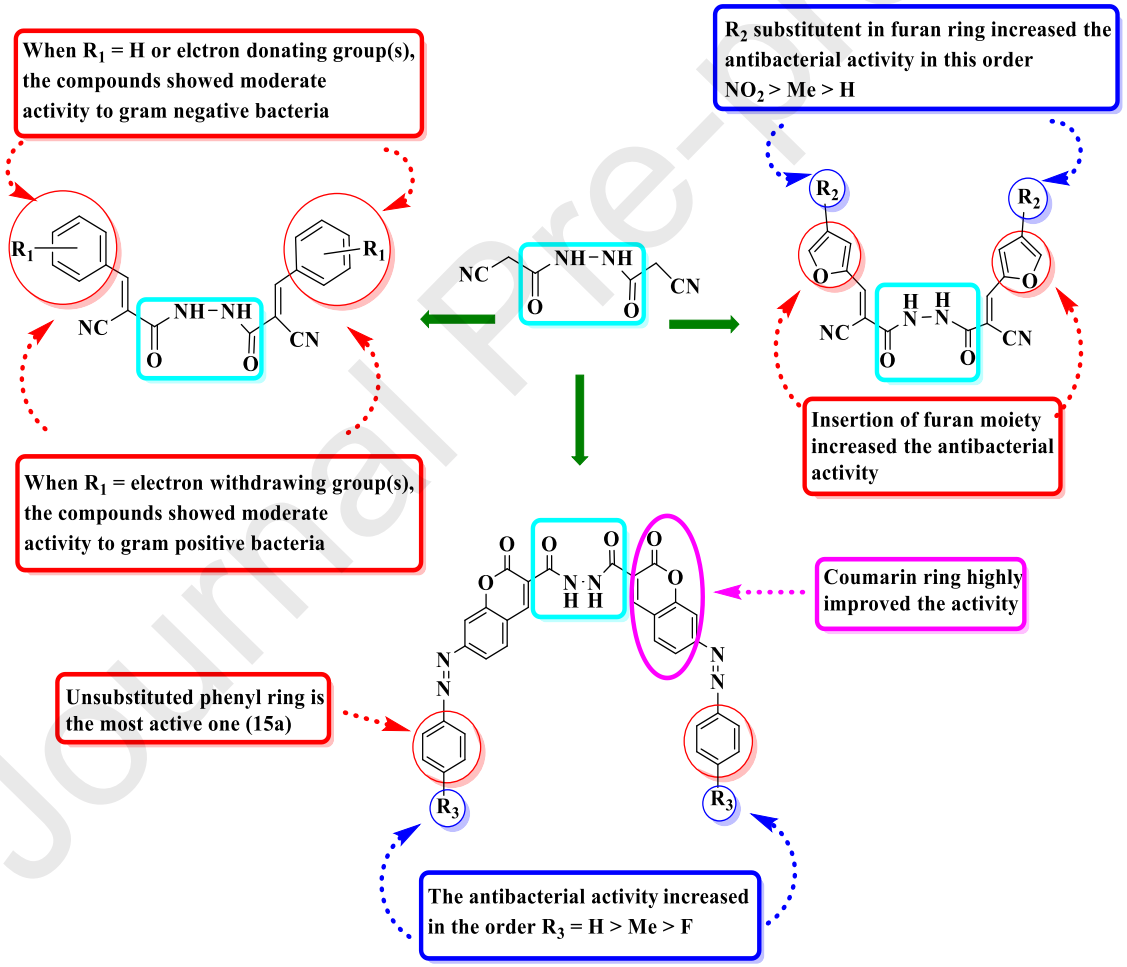
Table 1. Antibacterial activity of some tested compounds 3, 5a, 5b, 5d, 5e, 5h, 5i, 5j, 5k, 14b,14c, 14d, 14e, 15a, 15b, 15c, 15d, 15e, 17c, 17d and **17e against gram positive, gram negative bacteria and fungi.**

Compounds no.	Diameter inhibition zone in mm (% activity index)					
	Gram negative bacteria			Gram positive bacteria		Fungi
	***Escherichia coli* (ATCC:3008)**	***Klebsiella pneumonia* (ATCC:4415)**	***Pseudomonas aeruginosa* (ATCC:27853)**	***Staphylococcus aureus* (ATCC:6538)**	***Streptococcus mutans* (ATCC:25175)**	***Candida albicans* (ATCC:10231)**
3	NA	11.7 ± 0.5 (46%)	NA	13.7 ± 0.6 (65%)	12.7 ± 0.5 (43%)	NA
5a	14.8 ± 0.5 (54%)	19.9 ± 0.6 (79%)	NA	NA	14.9 ± 0.5 (51%)	NA
5b	13.7 ± 0.5 (50%)	19.7 ± 0.6 (78%)	NA	NA	14.7 ± 0.5 (50%)	NA
5c	NA	NA	NA	15.8 ± 0.5 (75%)	14.2 ± 0.5 (48%)	NA
5d	14.6 ± 0.5 (54%)	18.3 ± 0.6 (73%)	NA	NA	NA	10.6 ± 0.5 (50%)
5e	NA	NA	NA	13.3 ± 0.6 (63%)	12.5 ± 1.0 (43%)	NA
5h	13.8 ± 0.5 (51%)	NA	14 ± 0.5 (48%)	12 ± 0.5 (57%)	19.8 ± 0.5 (68%)	NA
5i	17.9 ± 0.5 (66%)	NA	NA	15 ± 0.5 (71%)	25.1 ± 0.5 (86%)	NA
5j	26.6 ± 0.6 (98%)	23.6 ± 0.6 (94%)	16.6 ± 0.5 (57%)	36.3 ± 0.6 (171%)	16.6 ± 0.6 (57%)	11.6 ± 0.6 (55%)
5k	24.6 ± 0.6 (91%)	24.3 ± 0.6 (97%)	NA	NA	NA	NA
14a	NA	NA	NA	NA	NA	NA
14b	NA	NA	NA	NA	NA	NA
14c	NA	NA	NA	NA	NA	NA
14d	NA	NA	NA	15.3 ± 0.5 (72%)	10.6 ± 0.5 (36%)	11.6 ± 0.5 (55%)
14e	NA	NA	NA	NA	NA	NA
15a	30 ± 0.5 (111%)	16 ± 0.5 (64%)	NA	27 ± 0.5 (128%)	37 ± 0.5 (127%)	22 ± 0.6 (104%)
15b	NA	15.6 ± 0.5 (62%)	NA	28.6 ± 0.6 (136%)	20.3 ± 0.6 (70%)	15.6 ± 0.5 (74%)
15c	NA	NA	NA	16.6 ± 0.6 (79%)	13.3 ± 0.5 (45%)	10.6 ± 0.5 (50%)
15d	NA	NA	NA	20.6 ± 0.6 (98%)	14.6 ± 0.5 (50%)	NA
15e	NA	12.6 ± 0.5 (50%)	NA	25.3 ± 0.6 (120%)	15.3 ± 0.5 (52%)	12.6 ± 0.5 (60%)
17c	NA	10.7 ± 0.5 (42%)	NA	NA	NA	NA

17d	NA	12.5 ± 0.5 (50%)	NA	NA	NA	NA
17e	NA	11.3 ± 0.5 (45%)	NA	NA	NA	NA
Gentamicin	27±0.5 (100%)	25±0.5 (100%)	29±0.5 (100%)	-	-	-
Ampicillin	-	-	-	21±0.1 (100%)	29±0.5 (100%)	-
Nystatin	-	-	-	-	-	21±0.5

- Zone of inhibition is expressed in the form of mean± standard deviation (mm).
- NA: No activity
- Well diameter (6mm).
- 100 µ l was tested.

2.2.2. Structure activity relationships (SAR)



It was found that the parent compound **3** has a moderate antibacterial activity against *K. pneumonia*, *S. aureus* and *S. mutans* with activity index 46, 65 and 43%, respectively. Introducing phenyl groups in the parent compound **3** as in compound **5a**,

resulted in an increase in the antibacterial activity towards both *K. pneumonia* and *S. mutans* with activity index 79 and 51%, respectively. The introduction of one electron donating group in each phenyl ring as in compound **5b**, the antibacterial activity decreased slightly towards *E. coli* (activity index 50%). On the other hand, the presence of two electron donating groups in each phenyl group like compound **5d** resulted in a moderate activity to *C. albicans* (activity index 50%).

Introducing one or two electron withdrawing groups in each phenyl group such as **5c** or **5e**, the activity against *E. coli* and *K. pneumonia* disappeared. But, they have activity to *S. aureus* and *S. mutans* with activity index 75 and 48% for **5c** and 63 and 43% for **5e**, respectively.

In compounds **5h** and **5i** containing the hetero-moiety thiophene and furan respectively, effected an increase of the antibacterial activity than the starting compound **3**. Increased antibacterial activity was observed on using substituted furan as in **5j** and **5k**. Thus, compound **5j** have a high activity towards all types of bacteria and fungi especially, *S. aureus* and *E. coli* (activity index 171 and 98%, respectively). Whereas, **5k** was active only to *E. coli* and *K. pneumonia* (activity index 91 and 97%, respectively).

Another category of synthesized compounds (imino coumarins) **14a-e** showed no activity against all types of the studied bacteria except for **14d** which exhibited activity to *S. aureus*, *S. mutans* and *C. albicans*. On the other hand, oxo-coumarins **15a-e** showed much better antibacterial activity than the imino-coumarins **14a-e**. Compound **15a** which contains unsubstituted azo phenyl group in the coumarin moiety have a strong activity to all types of bacteria and fungi except *P. aeruginosa*. Compound **15b** (methyl derivative) have activity index 62, 136, 70 and 74% with respect to *K. pneumonia*, *S. aureus*, *S. mutans* and *C. albicans*, respectively. Finally, azo derivatives **17c**, **17d** and **17e** have moderate activity against *K. pneumonia* only, while being inactive towards the other studied bacteria.

2.2.3. Minimum inhibitory concentration (MIC)

MIC was carried out for compounds **5c**, **5j**, **15a**, **15b**, **15c**, **15d** and **15e** with high antibacterial activity against *S. aureus, S. mutans, E. coli and K. pneumonia*. The results presented in Table 2 indicated that compound **5j** with high toxicity against *S. aureus* has the best minimum inhibitory concentration (MIC) against *s. aureus* (7.8 μg/ml), compared to the other tested compounds which is also better than MIC of the standard drug ampicillin (MIC = 62.5 μg/ml). Also, the MIC of compounds 1**5b** and 1**5e** with high toxicity against *s. aureus* is equal to that of the standard ampicillin (MIC = 62.5 μg/ml). The MIC of compound **15a** with the highest toxicity against *S. mutans* is 31.25 μg/ml and it is better than MIC of the standard drug ampicillin (MIC = 62.5 μg/ml). Regarding results in Table 3, it showed that both compounds **5j** and **5k** have MIC values against *K. pneumonia* which are equal to the standard gentamicin (MIC = 62.5 μg/ml). Also, **5j** is the only compound which has MIC value with respect to *P. aeruginosa* (MIC = 250 μg/ml). Moreover, compound **15a** has MIC value against *E. coli* which is equal to that of gentamicin (MIC = 31.25 μg/ml).

Table 2. Minimum inhibitory concentration of some tested compounds 5c, 5j, 15a, 15b, 15c, 15d and 15e on Gram positive bacteria.

Minimum inhibitory concentration (MIC) in μg/ml		
Gram positive bacteria		
Compound no.	***Staphylococcus aureus (ATCC:6538)***	***Streptococcus mutans (ATCC:25175)***
5c	**125**	**-**
5j	**7.8**	**250**
15a	**125**	**31.25**
15b	**62.5**	**250**
15c	**125**	**-**
15d	**250**	**-**
15e	**62.5**	**250**
Ampicillin	**62.5**	**62.5**

- Stock concentration 1 mg/ml

- Concentrations unit of MIC are represented as μg/ml

Table 3. Minimum inhibitory concentration of some tested compounds 5d, 5j, 5K, 15a and 15b on Gram negative bacteria.

Minimum inhibitory concentration (MIC) in µg/ml			
Gram negative bacteria			
Compound no.	***Escherichia coli (ATCC:9637)***	***Klebsiella pneumonia (ATCC:10031)***	***Pseudomonas aeruginosa (ATCC:27853)***
5d	**250**	**125**	**-**
5j	**125**	**62.5**	**250**
5k	**500**	**62.5**	**-**
15a	**31.25**	**250**	**-**
15b	**-**	**500**	**-**
Gentamicin	**31.25**	**62.5**	**125**

- Stock concentration 1 mg/ml

- Concentrations unit of MIC are represented as µg/ml

2.2.4. Enzyme inhibition of DNA gyrase and topoisomerase IV

DNA gyrase and topoisomerase IV inhibition activity was investigated for the most potent cytotoxic active compounds **5j**, **5k**, **15a**, **15b** and **15e** using methotrexate as a reference drug. The results deduced from Table 4 showed that all tested compounds exhibited potent inhibitory effect towards DNA gyrase and topoisomerase IV. Compound **15a** can strongly inhibit the two enzymes (IC_{50} = 27.30 and 25.52 µM respectively), compared to methotrexate. This means that the tested compounds may induce their inhibitory effect by binding to the enzymes active site causing inhibition of their activity which affected bacterial cell growth. This may be due to the similarity of structure **15a** to coumermycin A1 (Fig.2).

The data listed in Table 5 showed that methotrexate have the highest inhibitory effect on DNA gyrase and topo IV (92 and 94.7%, respectively). Among the tested compounds, it was found that compound **15a** have inhibitory effect on DNA gyrase and topoisomerase IV higher than the other tested compounds. Compound **15a** effected inhibition percent 88.9 and 93.7 followed by **5k** 86.4 and 88.3 of DNA gyrase and topoisomerase IV, respectively.

Table 4.IC_{50} (µM) of the tested compounds 5j, 5k, 15a, 15b, 15e and methotrexate on DNA gyrase and topoisomerase IV.

Compound no.	IC_{50} (µM) on DNA gyrase	IC_{50} (µM) on topoisomerase IV
5j	33.40±1.56	44.92±2.95
5k	30.34±1.82	33.98±2.08
15a	27.30±1.66	25.52±1.39
15b	30.96±1.44	34.45±1.86
15e	51.10±2.39	46.15±3.11
Methotrexate	29.01±1.27	23.55±1.71

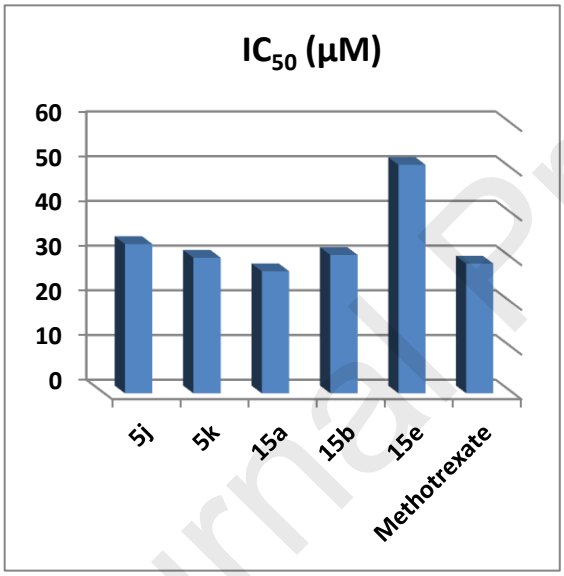


Fig. 5. IC_{50} (µM) on DNA gyrase

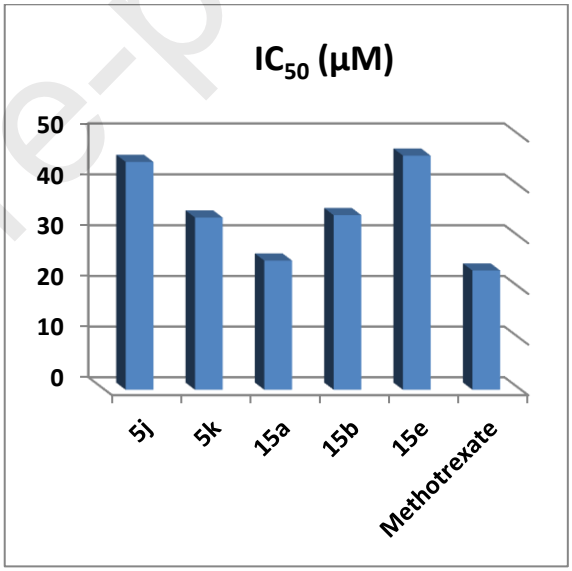


Fig. 6. IC_{50} (µM) on topoisomerase IV

Table 5. Inhibition percentage of the tested compounds 5j, 5k, 15a, 15b, 15e and methotrexate on DNA gyrase and topoisomerase IV.

Code	conc µM	log conc	%inhibition of DNA gyrase	%inhibition of topoisomerase IV
Methotrexate	100	2	92.00836	94.73674
	50	1.69897	70.08258	71.61601
	25	1.39794	45.49292	45.29972
	12.5	1.09691	19.87869	35.90105
5j				
	100	2	85.35236	85.52604
	50	1.69897	60.45163	63.9091
	25	1.39794	43.03395	32.95246
	12.5	1.09691	15.37059	10.71261
5k				
	100	2	86.47569	88.34565
	50	1.69897	62.09094	65.60086
	25	1.39794	48.1568	35.5251
	12.5	1.09691	25.00154	16.16384
15a				
	100	2	88.93465	93.79688
	50	1.69897	68.53469	82.14252
	25	1.39794	44.66446	54.51042
	12.5	1.09691	29.83598	17.66763
15b				
	100	2	80.7381	81.59155
	50	1.69897	67.82853	60.84304
	25	1.39794	36.68163	38.22249
	12.5	1.09691	25.41136	22.3101
15e				
	100	2	71.51698	85.90199
	50	1.69897	48.36171	51.69082
	25	1.39794	23.97697	13.34424
	12.5	1.09691	14.75585	6.577191

2.2.5. Agarose gel electrophoresis.

Investigation the influence of compounds **5j**, **5k**, **15a**, **15b**, **15e** and methotrexate at different concentrations of 100, 50, 25, 12.5 and 7 µM on the enzymes using the supercoiling assay for DNA gyrase and the decantation assay for topo IV (Fig 7 and 8). The first lane, labeled (-) contains a control assay without enzymes while the second lane, labeled (+) contains enzyme assays (DNA gyrase or topoisomerase IV) which indicates supercoiled DNA in case of DNA gyrase and decanted DNA in case of topoisomerase IV [6,40]. At higher drug concentrations, inhibition of the enzyme is

very high like the negative control and the enzymes activity increase by decreasing the drug concentration. Compounds **15a** and **5k** have high ability to inhibit the enzymes activity which reflected on the bands pattern of the gel, while **15e** is the lowest compound.

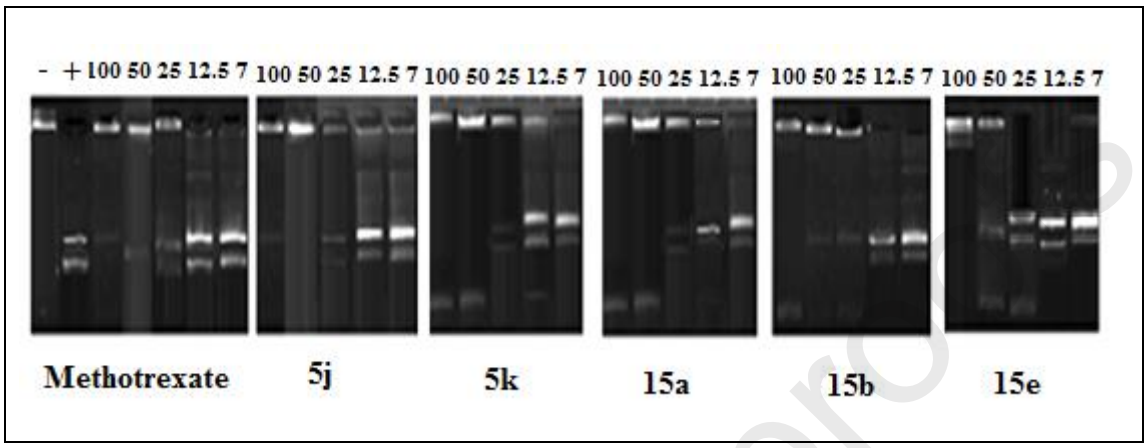


Fig. 7. Electrophoretic analysis of bacterial DNA gyrase assays with the Inhibitory effect of 5j, 5k, 15a,15b, 15e.

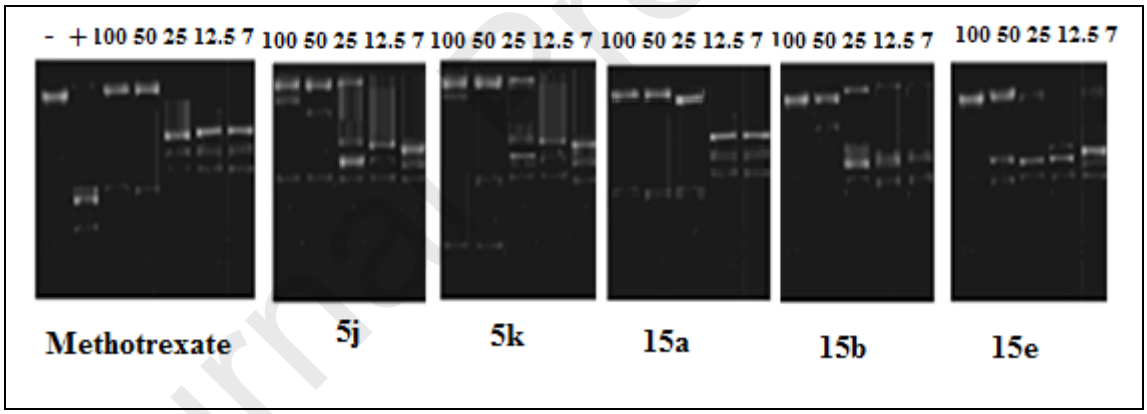


Fig. 8. Electrophoretic analysis of bacterial topoisomerse IV assays with the Inhibitory effect of 5j, 5k, 15a,15b, 15e.

2.2.6. Molecular modeling and docking study

Docking simulation for the newly synthesized compounds **5j**, **5k**, **15a**, **15b**, **15e** and methotrexate was carried out using MOE 2009.10 software. The crystal structure of the selected protein was downloaded from a protein data bank (http://www. PDB.org) with pdb code = 6ENH (Fig 9). Refinement of the crude pdb structure was performed, and then saved as a moe file to be used for docking simulation. The 2D structures of

the selected compounds **5j**, **5k**, **15a**, **15b**, **15e** and methotrexate were converted into their 3D forms using Chem 3D ultra 12.0 software and then the energy was minimized and saved as mol. file. Docking simulation was performed as in the MOE 2009.10 manual; all these compounds were docked into the same active site of the ligand and the binding affinities were calculated. The results of the docking simulation together with the measured affinities and distances are shown in Table 6.

According to the docking simulation results, compounds **5j**, **5k**, **15a**, **15b**, **15e** and methotrexate have binding energies of -8.6143, -10.2332, -7.1273, -8.1949, -9.1186 and -13.4633 kcal/mole, respectively. Docking of methotrexate with the active site of the selected protein showed three hydrogen bonds, two are formed between oxygen and Arg 123 with bond length (1.95 Å) and CO with Arg 123 (2.09 Å). The third one between NH_2 and Asp 70 (1.97 Å) (Fig. 10). The mode of interactions for compounds **15a** and **5k** which appeared to have the highest potency against DNA gyrase and topoisomerase IV are illustrated. Thus compound **15a** showed four interactions three of them are hydrogen bond interactions and one arene-cation interaction. All hydrogen bonds are between CO linked to Arg 133 and Arg 73 (1.89, 2.32 and 2 Å) respectively, and the arene-cation interaction is between phenyl ring and Arg 73 (Fig. 13). Also, compound **5k** exhibited three hydrogen bond between CN and Arg 73, Gly 74 and Thr 164 (2.49, 2.07 and 2.53 Å, respectively) (Fig. 12). Moreover, the molecular docking of compound **15b** with active site of enzyme presented three interactions two of them for arene-cation and one for hydrogen bond between CO linked to Asn 43 (2.08 Å) (Fig. 14). Compound **5j** have a moderate activity towards the enzymes which showed two interactions, a hydrogen bonding between CO and Arg 73 (2.26 Å) and arene-cation interaction between furan ring and Arg 73 (Fig. 11). Molecular docking of compound **15e** which has the lowest cytotoxic activity showed only one hydrogen bonding interaction between CO and Arg 73 (2.11 Å) (Fig. 15).

Table 6. The protein-ligand interactions of some tested compounds and methotrexate (standard drug) with 6ENH active site pocket.

Compounds	Binding energy (S) Kcal/mol	Groups of the ligand involved in the interaction	Amino acids involved in the interaction	Type of the bond and length of Hydrogen bonds Å
Methotrexate	-13.4633	O^-	Arg 123	Basic side chain acceptor1.95 Å
		C=O	Arg 123	Basic side chain acceptor 2.09 Å
		NH_2	Asp 70	Acidic side chain donor 1.97 Å
5j	-8.6143	C=O	Arg 73	Basic side chain acceptor 2.26 Å
		Furan ring	Arg 73	Basic (Arene-cation interaction)
5k	-10.2332	CN	Arg 73	Basic side chain acceptor 2.49 Å
		CN	Gly 74	Polar backbone acceptor 2.07 Å
		CN	Thr 164	Polar side chain acceptor 2.53 Å
15a	-7.1273	C=O	Arg 133	Basic side chain acceptor 1.89 Å
		C=O	Arg 133	Basic side chain acceptor 2.32 Å
		C=O	Arg 73	Basic side chain acceptor 2 Å
		Phenyl ring	Arg 73	Basic (Arene-cation interaction)
15b	-8.1949	C=O	Asn 43	Polar side chain acceptor 2.08 Å
		Phenyl ring	Arg 133	Basic (Arene-cation interaction)
		Phenyl ring	Arg 73	Basic (Arene-cation interaction)
15e	-9.1186	C=O	Arg 73	Basic side chain acceptor 2.11 Å

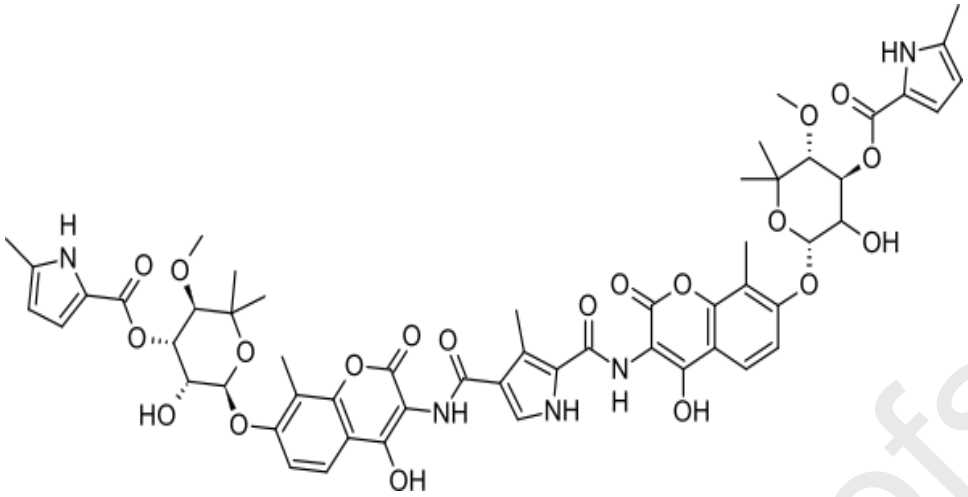
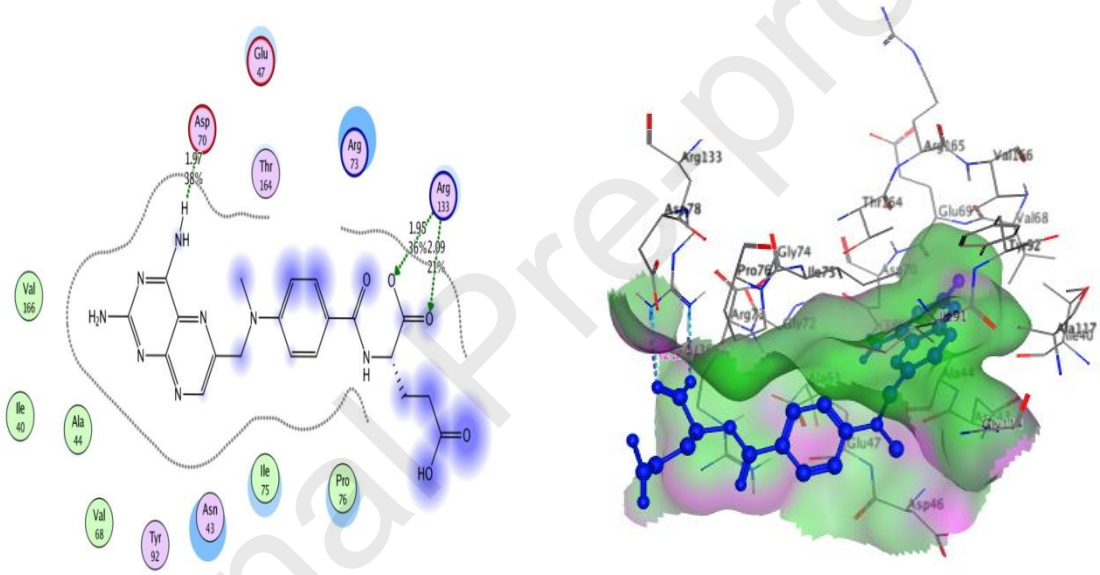
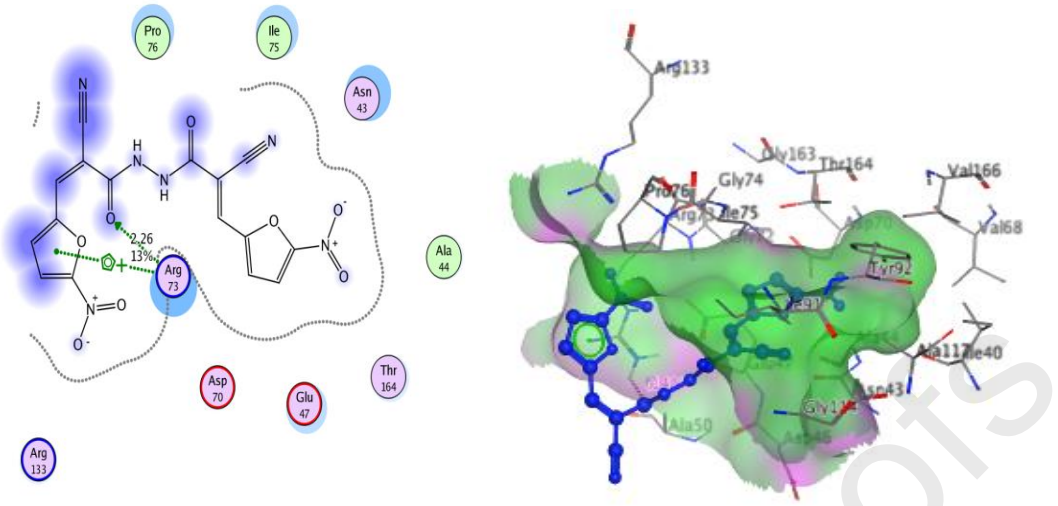


Fig. **9**. Structure of 6ENH.



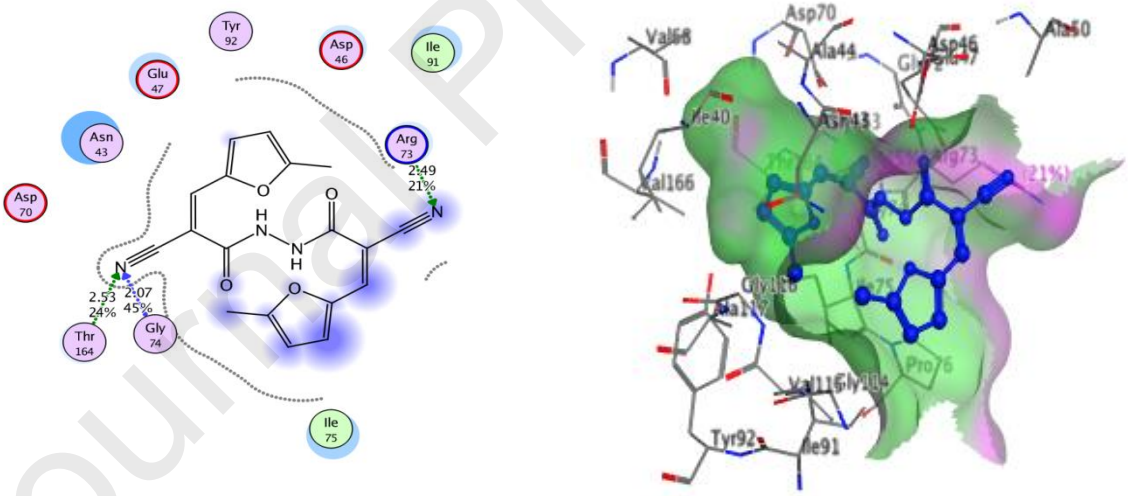
2D structure 3D structure

Fig. **10**. Binding pattern of methotrexate (2D and 3D) structure with the active site of DNA gyrase and topoisomerase IV enzymes (PDB code = 6ENH).



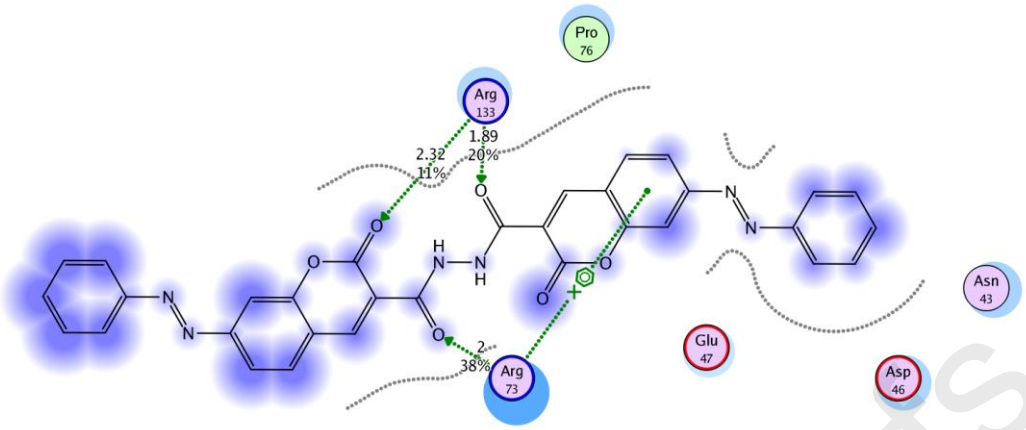
2D structure 3D structure

Fig. **11**. Binding pattern of compound **5j** (2D and 3D) structure with the active site of DNA gyrase and topoisomerase IV enzymes (PDB code = 6ENH).

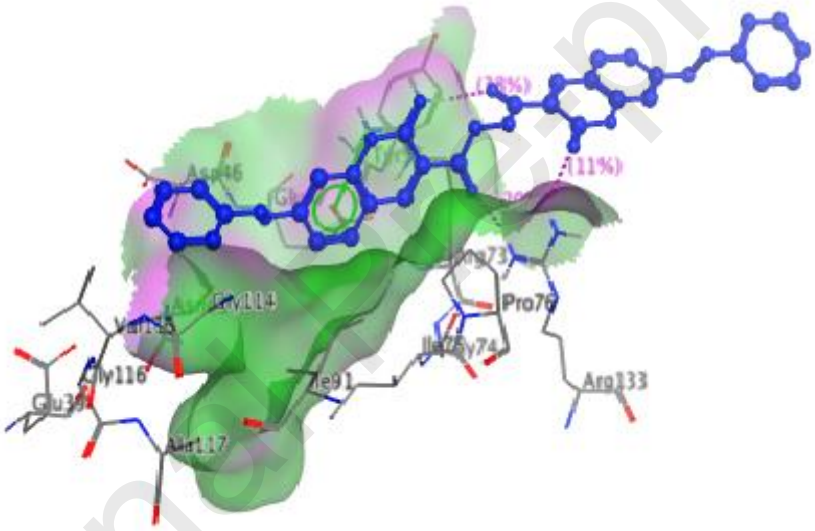


2D structure 3D structure

Fig. **12**. Binding pattern of compound **5k** (2D and 3D) structure with the active site of DNA gyrase and topoisomerase IV enzymes (PDB code = 6ENH).

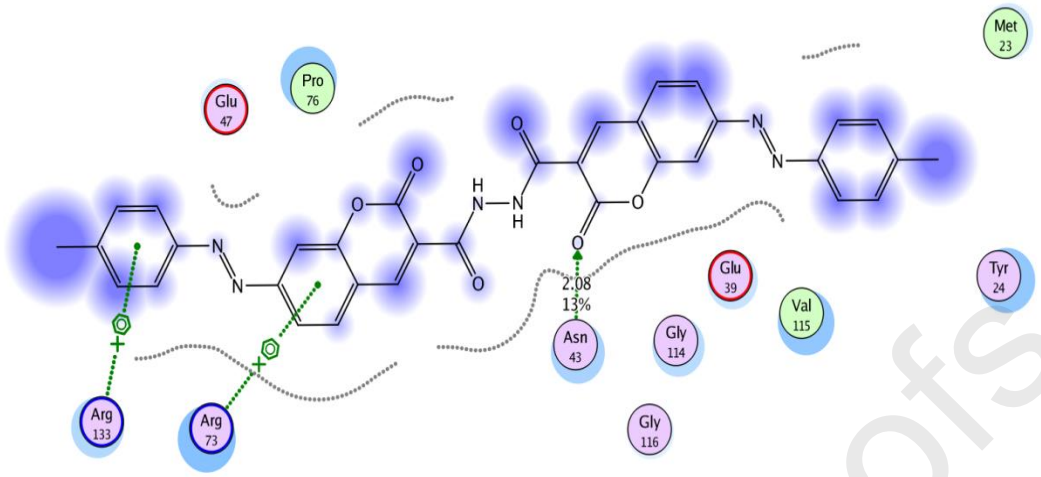


2D structure

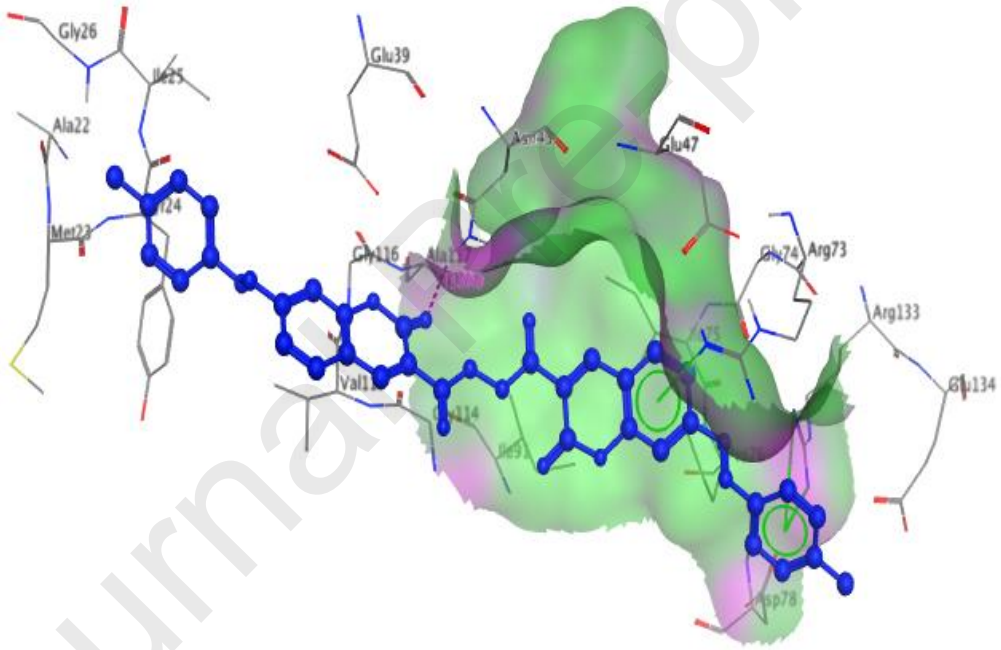


3D structure

Fig. **13**. Binding pattern of compound **15a** (2D and 3D) structure with the active site of DNA gyrase and topoisomerase IV enzymes (PDB code = 6ENH).

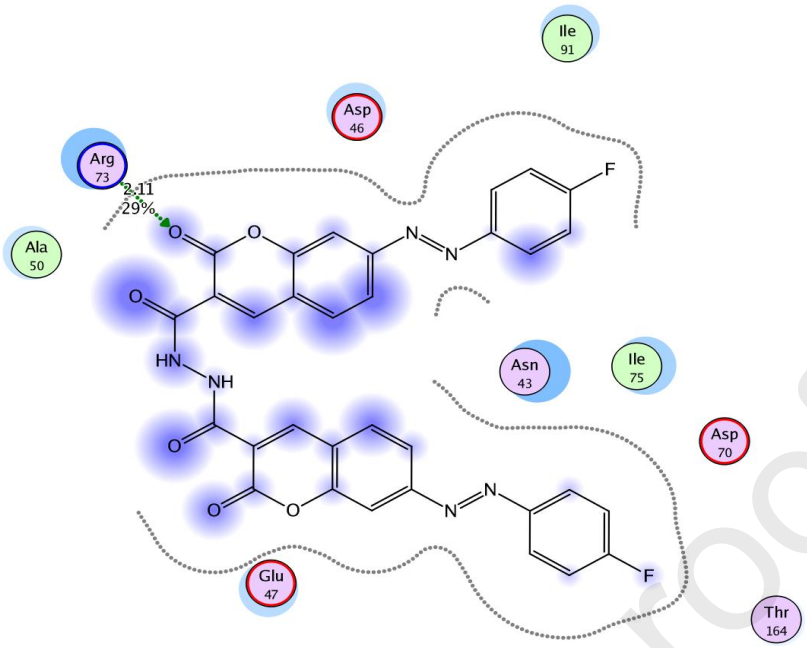


2D structure

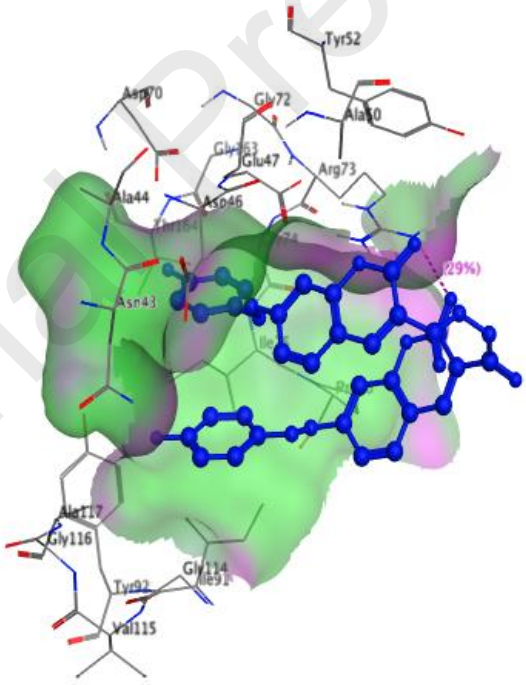


3D structure

Fig. **14**. Binding pattern of compound **15b** (2D and 3D) structure with the active site of DNA gyrase and topoisomerase IV enzymes (PDB code = 6ENH).



2D structure



3D structure

Fig. **15**. Binding pattern of compound **15e** (2D and 3D) structure with the active site of DNA gyrase and topoisomerase IV enzymes (PDB code = 6ENH).

3. Conclusion

Some novel bis(cyanoacetyl)hydrazines and bis-coumarin derivatives were synthesized and evaluated *in vitro* against bacterial cell lines. Results revealed that the bis-coumarin derivative **15a** is the most potent inhibitor of DNA gyrase and topoisomerase IV (essential for bacterial cell growth), which can be attributed to its structural similarity to 6ENH native ligand.

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were determined on an Electrothermal (9100) apparatus and are uncorrected. The IR spectra were recorded as KBr pellets on a Perkin Elmer 1430 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded in deuterated dimethylsulfoxide at 300 and 75 MHz, respectively, on a Varian Gemini NMR spectrometer using tetramethylsilane as internal reference and the results are expected as δ value. Mass spectra were taken on a Shimadzu GCMS-QP 1000 Ex mass spectrometer at 70 eV. Elemental analyses were carried out at the Microanalyses Center of Cairo University, Giza-Egypt. Antibacterial activity and MIC carried out in laboratory of Biochemistry at Faculty of Science-Cairo University-Egypt. Enzyme inhibition tests were carried out in Science Way laboratory in Cairo. Physical and spectral data of all newly synthesized compounds are found in supplementary information 1.

4.1.2. 2-Cyano-*N'*-(2-cyanoacetyl)acetohydrazide (3).

A mixture of 2-cyanoacetohydrazide (**1**) (0.01mol) and 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile (**2**) (0.01mol) was heated in glacial acetic acid in water bath at 40 ºC for one hour, then allowed to cool. The solid collected by filtration and recrystallized from an ethanol-dioxane mixture.

White crystals; yield 90%; mp. 195 ºC (lit., 200-202 ºC) [41]; IR (KBr) 3203, 3063 (NH), 2263 (CN), 1617 (CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 3.75 (s, 4H, 2CH$_2$), 10.38 (s, 2H, 2NH). Anal. Calcd. $C_6H_6N_4O_2$ (166.14): C, 43.38; H, 3.64; N, 33.72. Found: C, 43.58; H, 3.46; N, 33.94 %.

4.1.3. Synthesis of compounds 5a-k and 10a,b.

A mixture of 2-cyano-*N'*-(2-cyanoacetyl)acetohydrazide (**3**) (0.01mol) and the appropriate aromatic aldehyde **4a-k** and / or **8a,b** (0.01mol) was ground in presence of sodium hydroxide (0.01mol). The ice cold water was poured into the mixture, then the solid was filtered off and washed well with water, then recrystallized from *N*,*N*-dimethylformamide.

4.1.3. 1. 2-Cyano-*N'*-(2-cyano-3-phenylacryloyl)-3-phenylacrylohydrazide (5a).

Yellow crystals; yield 60%; mp. 285 ºC; IR (KBr) 3431, 3296 (NH), 2212 (CN), 1639 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.29-7.53 (m, 10H, Ar), 7.94 (s, 2H, 2CH), 10.62 (s, 2H, 2NH); m/z = 342 (M^+, 2.99%), 239 (3.35%), 186 (4.61%), 165 (29.4%), 120 (13.3%), 110 (12.6%), 103 (22.2%), 91 (54.4%), 83 (26.4%), 60 (47.6%), 57 (94.4%), 55 (100%), 51 (55.5%). Anal. Calcd. $C_{20}H_{14}N_4O_2$ (342.11): C, 70.17; H, 4.12; N, 16.37. Found: C, 70.35; H, 4.30; N, 16.15 %.

4.1.3.2. 2-Cyano-*N'*-(2-cyano-3-(2-methoxyphenyl)acryloyl)-3-(2-methoxyphenyl) acrylohydrazide (5b).

Orange crystals; yield 58%; mp. 250 ºC; IR (KBr) 3432 (NH), 2210 (CN), 1649 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 3.90 (s, 6H, $2OCH_3$), 7.08-8.02 (m, 8H, Ar), 8.38 (s, 2H, 2CH), 10.52 (s, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ = 56.2, 112.2, 113.1, 118.3, 121.9, 128.4, 133.4, 136.9, 158.4; m/z = 402 (M^+, 1.01%), 375 (1.47%), 285 (2.85%), 215 (12.6%), 186 (20.3%), 160 (11.9%), 121 (100%), 111 (10.8%), 99 (10.3%), 89 (16.4%), 77 (20.0%), 69 (13.0%). Anal. Calcd. $C_{22}H_{18}N_4O_4$ (402.13): C, 65.66; H, 4.51; N, 13.92. Found: C, 65.49; H, 4.71; N, 14.15 %.

4.1.3.3. 2-Cyano-*N'*-(2-cyano-3-(2,5-dimethoxyphenyl)acryloyl)-3-(2,5-dimethoxyphenyl)acrylohydrazide (5c).

Orange crystals; yield 69%; mp. 235 ºC; IR (KBr) 3428, 3288 (NH), 2209 (CN), 1642 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 3.75 (s, 6H, $2OCH_3$), 3.83 (s, 6H, $2OCH_3$), 6.76-7.64 (m, 6H, Ar), 8.32 (s, 2H, 2CH), 10.42 (s, 2H, 2NH). Anal. Calcd. $C_{24}H_{22}N_4O_6$ (462.15): C, 62.33; H, 4.80; N, 12.12. Found: C, 62.52; H, 4.62; N, 12.36 %.

Orange crystals; yield 79%; mp. >300 ºC; IR (KBr) 3433, 3303 (NH), 2211 (CN), 1642 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.38 (s, 6H, $2CH_3$), 6.40-7.15 (m, 4H, Ar), 7.75 (s, 2H, 2CH), 10.44 (s, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ = 14.2, 110.7, 115.8, 118.3, 129.0, 135.4, 148.6, 155.8, 164.3; m/z = 350 (M^+, 0.3%), 328 (1.5%), 313 (1.2%), 270 (15.8%), 268 (21.8%), 139 (11.6%), 125 (20.1%), 111 (30.6%), 97 (46.1%), 83 (54.3%), 71 (59.2%), 57 (100%), 55 (66.5%). Anal. Calcd. $C_{18}H_{14}N_4O_4$ (350.10): C, 61.71; H, 4.03; N, 15.99. Found: C, 61.88; H, 4.20; N, 15.75 %.

4.1.3.12. 2-Imino-*N'*-(2-imino-2*H*-chromene-3-carbonyl)-2*H*-chromene-3-carbohydrazide (10a).

Orange crystals; yield 62%; mp. >300 ºC; IR (KBr) 3433 (NH), 1669 (CO), 1611 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 6.72-7.57 (m, 8H, Ar), 9.00 (s, 2H, 2CH), 10.25 (s, 2H, 2NH), 10.68 (s, 2H, 2NH); m/z = 374 (M^+, 4.88%), 357 (18.13%), 305 (49.21%), 268 (100%), 240 (58.10%), 223 (41.60%), 214 (40.58%), 186 (15.57%), 143 (72.91%), 118 (41.34%), 107 (40.06%), 88 (54.10%), 78 (52.40%), 63 (95.82%), 57 (35.58%), 51 (84.20%). Anal. Calcd. $C_{20}H_{14}N_4O_4$ (374.10): C, 64.17; H, 3.77; N, 14.97. Found: C, 64.34; H, 3.60; N, 14.73 %.

4.1.3.13. 6-Hydroxy-*N'*-(6-hydroxy-2-imino-2*H*-chromene-3-carbonyl)-2-imino-2*H*-chromene-3-carbohydrazide (10b).

Red crystals; yield 71%; mp. >300 ºC; IR (KBr) 3430 (NH), 3399 (OH), 1662 (CO), 1580 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 6.70-7.60 (m, 6H, Ar), 9.12 (s, 2H, 2CH), 9.92 (s, 2H, 2OH), 10.30 (s, 2H, 2NH), 10.64 (s, 2H, 2NH). Anal. Calcd. $C_{20}H_{14}N_4O_6$ (406.09): C, 59.12; H, 3.47; N, 13.79. Found: C, 58.93; H, 3.27; N, 13.54 %.

4.1.4. Synthesis of compounds 11a,b.

Compounds **10a,b** (0.01mol) were refluxed in dioxane containing few drops of conc. HCl for three hours. The solid was collected by filtration, and recrystallized from *N*,*N*-dimethylformamide.

4.1.4.1. 2-Oxo-N'-(2-oxo-2*H*-chromene-3-carbonyl)-2*H*-chromene-3-carbohydrazide (11a).

Orange crystals; yield 59%; mp. >300 ºC; IR (KBr) 3438 (NH), 1680 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 6.70-7.58 (m, 8H, Ar), 8.89 (s, 2H, 2CH), 10.68 (s, 2H, 2NH).

Anal. Calcd. $C_{20}H_{12}N_2O_6$ (376.07): C, 63.83; H, 3.21; N, 7.44. Found: C, 64.00; H, 3.40; N, 7.20 %.

4.1.4.2. 7-Hydroxy-*N'*-(7-hydroxy-2-oxo-2*H*-chromene-3-carbonyl)-2-oxo-2*H*-chromene-3-carbohydrazide (11b).

Red crystals; yield 61%; mp. >300 ºC; IR (KBr) 3432 (NH), 3397 (OH), 1668 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 6.74-7.62 (m, 6H, Ar), 9.12 (s, 2H, 2CH), 9.90 (s, 2H, 2OH), 10.64 (s, 2H, 2NH). Anal. Calcd. $C_{20}H_{12}N_2O_8$ (408.06): C, 58.83; H, 2.96; N, 6.86. Found: C, 59.00; H, 2.79; N, 7.10 %.

4.1.5. Synthesis of compounds 14a-e.

A mixture of 2-cyano-*N'*-(2-cyanoacetyl)acetohydrazide (**3**) (0.01mol), azosalicylaldehydes **12a-e** (0.01mol) and sodium hydroxide (0.01mol) was ground. The solid so formed was filtered off and washed with water, then recrystallized from *N*,*N*-dimethylformamide.

4.1.5.1. 2-Imino-*N'*-(2-imino-7-(phenyldiazenyl)-2*H*-chromene-3-carbonyl)-7-(phenyl diazenyl)-2*H*-chromene-3-carbohydrazide (14a).

Brown crystals; yield 77%; mp. >300 ºC; IR (KBr) 3425 (NH), 1668 (CO), 1570 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 6.56-7.82 (m, 16H, Ar), 9.15 (s, 2H, 2CH), 10.05 (s, 2H, 2NH), 10.73 (s, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ = 110.5, 114.6, 115.5, 118.3, 123.0, 129.0, 129.9, 130.9, 152.7, 153.3, 154.8, 160.4, 165.9); m/z = 582 (M^+, 0.45%), 423 (0.84%), 368 (2.52%), 330 (2.53%), 302 (4.44%), 226 (13.7%), 167 (5.62%), 149 (10.4%), 91 (47.5%), 77 (100%), 65 (64.5%), 57 (55.5%). Anal. Calcd. $C_{32}H_{22}N_8O_4$ (582.18): C, 65.97; H, 3.81; N, 19.23. Found: C, 66.16; H, 4.01; N, 19.00 %.

4.1.5.2. 2-Imino-*N'*-(2-imino-7-(*p*-tolyldiazenyl)-2*H*-chromene-3-carbonyl)-7-(*p*-tolyl diazenyl)-2*H*-chromene-3-carbohydrazide (14b).

Brown crystals; yield 78%; mp. >300 ºC; IR (KBr) 3429 (NH), 1664 (CO), 1523 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.36 (s, 6H, $2CH_3$), 7.28-7.83 (m, 14H, Ar), 8.24 (s, 2H, 2CH), 9.20 (s, 2H, 2NH), 10.42 (s, 2H, 2NH). Anal. Calcd. $C_{34}H_{26}N_8O_4$ (610.21): C, 66.88; H, 4.29; N, 18.35. Found: C, 66.67; H, 4.08; N, 18.60 %.

4.1.5.3. 2-Imino-*N′*-(2-imino-7-((4-methoxyphenyl)diazenyl)-2*H*-chromene-3-carbonyl)-7-((4-methoxyphenyl)diazenyl)-2*H*-chromene-3-carbohydrazide (14c).

Orange crystals; yield 80%; mp. >300 ºC; IR (KBr) 3429 (NH), 1663 (CO), 1599 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 3.83 (s, 6H, $2OCH_3$), 7.00-7.86 (m, 14H, Ar), 8.68 (s, 2H, 2CH), 9.12 (s, 2H, 2NH), 10.20 (s, 2H, 2NH); m/z = 642 (M^+, 0.13%), 508 (6.4%), 389 (1.4%), 305 (37.9%), 270 (39.9%), 255 (4.4%), 170 (4.4%), 135 (32.8%), 107 (100%), 77 (99.8%), 64 (38.8%). Anal. Calcd. $C_{34}H_{26}N_8O_6$ (642.20): C, 63.55; H, 4.08; N, 17.44. Found: C, 63.72; H, 4.28; N, 17.21 %.

4.1.6. Synthesis of compounds 15a-e.

Compounds **14a-e** (0.01mol) were refluxed in dioxane containing few drops of conc. HCl for three hours. The solid so formed was filtered off, and recrystallized from *N*,*N*-dimethylformamide.

4.1.6.1. 2-Oxo-*N′*-(2-oxo-7-(phenyldiazenyl)-2*H*-chromene-3-carbonyl)-7-(phenyl diazenyl)-2*H*-chromene-3-carbohydrazide (15a).

Brown crystals; yield 55%; mp. >300 ºC; IR (KBr) 3426 (NH), 1670 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.21-7.80 (m, 16H, Ar), 8.32 (s, 2H, 2CH), 10.46 (s, 2H, 2NH). Anal. Calcd. $C_{32}H_{20}N_6O_6$ (584.55): C, 65.75; H, 3.45; N, 14.38. Found: C, 65.95; H, 3.65; N, 14.60 %.

4.1.6.2. 2-Oxo-*N′*-(2-oxo-7-(*p*-tolyldiazenyl)-2*H*-chromene-3-carbonyl)-7-(*p*-tolyldiazenyl) -2*H*-chromene-3-carbohydrazide (15b).

Brown crystals; yield 58%; mp. >300 ºC; IR (KBr) 3429 (NH), 1620 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.36 (s, 6H, $2CH_3$), 7.28-7.83 (m, 14H, Ar), 8.24 (s, 2H, 2CH), 10.42 (s, 2H, 2NH). Anal. Calcd. $C_{34}H_{24}N_6O_6$ (612.60): C, 66.66; H, 3.95; N, 13.72. Found: C, 66.84; H, 4.15; N, 13.50 %.

4.1.6.2. 7-((4-Methoxyphenyl)diazenyl)-*N′*-(7-((4-methoxyphenyl)diazenyl)-2-oxo-2*H*-chromene-3-carbonyl)-2-oxo-2*H*-chromene-3-carbohydrazide (15c).

Orange crystals; yield 63%; mp. >300 ºC; IR (KBr) 3426 (NH), 1713 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 3.83 (s, 6H, $2OCH_3$), 7.10-7.88 (m, 14H, Ar), 8.66 (s, 2H, 2CH), 10.20 (s, 2H, 2NH). Anal. Calcd. $C_{34}H_{24}N_6O_8$ (644.17): C, 63.35; H, 3.75; N, 13.04. Found: C, 63.53; H, 3.55; N, 13.28 %.

4.1.7. Synthesis of compounds 17a-e.

A mixture of 2-cyano-*N'*-(2-cyanoacetyl)acetohydrazide (**3**) (0.01 mol), aromatic amines **16a-e** (0.01 mol) and sodium nitrite in presence of *p*-toluenesulfonic acid (0.01 mol) was ground in ice bath. The mixture stand in ice bath for one hour. The solid collected by filtration, washed with water and recrystallized from *N,N*-dimethylformamide.

4.1.7.1. 2,2'-(Hydrazine-1,2-diyl)bis(2-oxo-*N*-phenylacetohydrazonoylcyanide) (17a).

Orange crystals; yield 82%; mp. >300 ºC; IR (KBr) 3430 (NH), 2214 (CN), 1680 (CO), 1560 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.10-7.40 (m, 10H, Ar), 10.12 (s, 2H, 2NH), 11.64 (s, 2H, 2NH). Anal. Calcd. $C_{18}H_{14}N_8O_2$ (374.12): C, 57.75; H, 3.77; N, 29.93. Found: C, 57.55; H, 3.94; N, 29.70 %.

4.1.7.2. 2,2'-(Hydrazine-1,2-diyl)bis(2-oxo-*N*-(*p*-tolyl)acetohydrazonoylcyanide) (17b).

Red crystals; yield 80%; mp. >300 ºC; IR (KBr) 3435, 3225 (NH), 2220 (CN), 1668 (CO), 1552 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.29 (s, 6H, $2CH_3$), 7.17 (d, 4H, *J* = 8.1 Hz, 4CH), 7.61 (d, 4H, *J* = 8.4 Hz, 4CH), 10.12 (s, 2H, 2NH), 11.84 (s, 2H, 2NH); ^{13}C NMR (DMSO-d_6) δ = 20.2, 105.2, 110.9, 116.0, 129.4, 133.5, 139.6, 160.6; m/z = 402 (M^+, 100%), 373 (5.28%), 296 (5.76%), 283 (4.65%), 217 (36.52%), 119 (12.26%), 106 (25.76%), 91 (84.23%), 77 (25.09%), 65 (21.75%), 51 (5.54%). Anal. Calcd. $C_{20}H_{18}N_8O_2$ (402.16): C, 59.69; H, 4.51; N, 27.85. Found: C, 59.90; H, 4.30; N, 28.10 %

4.2. Biology

4.2.1 Antimicrobial assay

Agar well diffusion method utilized for the determination antimicrobial screening of some new prepared compounds. Some pathogenic types of bacteria like *S. aureus*, *S. mutans* (Gram positive bacteria bacteria), *E. coli*, *P. aeruginosa* and *k. pneumonia* (Gram negative bacteria) were used along with *C. albicans* (fungi) *in vitro* using nutrient agar medium. Standard drugs for Gram positive, Gram negative bacteria and fungi used in this investigation are Ampicillin, Gentamicin and Nystatin respectively,

using DMSO as solvent control. The concentration of tested compounds is 15 mg/ml against pathogenic bacteria and fungal strains which employed in this study.

4.2.2. Method of testing

Nutrient and saboured dextrose agar media were prepared, sterilized and poured on Petri dishes sterilized by McFarland 0.5 standard solution (1.5x 10^5CFU mL^{-1}). The used bacteria and fungi was prepared using normal sterile saline. OD of the prepared McFarland 0.5 solution should be adjusted to 0.13 utilizing spectrophotometer at 625 nm. Within 15 min. after adjusting the OD of the microbial suspension, 100 µL of the inoculums suspension was plated on the solidified agar surface, then covered and allow drying. Sterile borer was used to make wells of 6mm diameter. A micropipette was used to add 100 µL of the tested compound solutions. Finally, the plates were incubated for 24 h for bacteria and 48 h for fungi at 37 °C. This experiment was carried out in triplicate and zones of inhibition were measured in mm. scale.

4.2.3. Methodology of MIC

For each strain, three to five isolated colonies were selected from the fresh agar plate and were transferred into a tube containing 3-4 ml of sterile broth medium. The bacterial suspension was mixed well and incubated at 35-37 °C for 2-6 h. The turbidity of the bacterial suspension should be equal to or greater than the turbidity of a McFarland Standard 0.5. After that, 1 mg of the tested compound (antimicrobial agent) was dissolved in 1 ml DMSO and two-fold serial dilution was done using broth medium. A fixed volume of the prepared bacterial inoculum was added to each tube and incubated for at 37 °C 16-20 h. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye [42].

4.2.4. Enzyme inhibition

Detection of DNA gyrase by using DNA gyrase assay Kit Description (TG1003) from TopoGEN, Inc. 108 Aces Alley, Port Orange, Florida 32128, USA.

This kit facilitates the purification and characterization of type II enzymes (DNA gyrase) based upon decatentation of kinetoplast DNA (kDNA) and supercoiling of the resulting decatenated monomer kDNA species. The reaction products are resolved

using a novel gel system developed by TopoGEN that allows extremely rapid and unambiguous detection of gyrase activity. The kDNA assay detected poisons that stimulate DNA cleavage by gyrase. The decatenated DNA marker Opened Circular DNA (OC, 2.5 KB monomers with at least one nick) and some Closed Circular DNA (CC) DNA. Linear DNA produced during nuclease contamination. The degree of decatenation of kDNA by topoisomerase II or gyrase is proportional to the amount of enzyme and the length of incubation. The most active fractions will completely decatenate the kDNA. For screening of column fractions or other assays in which many reactions will be conducted, it is best to set up a master reaction mixture containing all reagents except the test fraction, then aliquoting this into the reaction tubes to which the fractions will be added. High concentrations of salt inhibit topoisomerases; therefore, test fractions should not contribute greater than 30 mM monovalent salt to the final reaction. If the protein fractions contain agents which alter the electrophoretic mobility of DNA it may be necessary to phenol/chloroform treat and/or ethanol precipitate samples before loading the gels.

Purified E. coli Topoisomerase IV Enzyme [TG2000EC……1, TG2000EC…..3, TG2000EC…..5] from TopoGEN, Inc. 108 Aces Alley, Port Orange, Florida 32128, USA.

TopoGEN offers purified Topo IV for use in all aspects of drug development and screening assays. The enzyme is very active in decatenating kDNA (TG2013) but will also relax plasmid DNA Topo IV is purified according to Peng and Marians.

1. Topo IV was overexpressed and purified. A single band on SDS--PAGE was detected by CB staining for each subunit. Cross contamination by topo I was assessed by assaying for relaxation of supercoiled DNA under conditions optimized for type I activity. Under these conditions, after 2 hours of incubation with pBR322, no relaxation products were detectable.

2. A test for nuclease contamination was carried out by assaying for the formation of linear kDNA and linear plasmid DNA. Incubations of 1 μg of catenated kDNA or supercoiled pUC19 DNA (4 hrs. at 37 °C in the presence of 10 mM $MgCl_2$) were performed. Linear DNA or breakdown products were not generated under these conditions.

3. The subunits are >95% pure based upon SDS--PAGE and certified to be endonuclease free.

4.2.5. Molecular docking Study

MOE version 2009.10 [43] used to docking study and all modeling calculations. Coumermycin A1 structure was downloaded from the protein data bank (PDB) [http://www.rcsb.org/PDB codes: 6ENH].

References

[1] R. J. Scheffler, S. Colmer, H. Tyman, A. L. Demain, V. P. Gullo, Antimicrobials, drug discovery and genome mining, *Appl. Microbiol. Biotechnol*. **97** (2013) 969-978.

[2] L. Zhang, X.M. Peng, G. L.V. Damu, R. X. Geng, C. H. Zhou, Comprehensive review in current developments of imidazole-based medicinal chemistry, *Med. Res. Rev*. **34** (2014) 340-437.

[3] X. M. Peng, G. L.V. Damu, C. H. Zhou, Current developments of coumarin compounds in medicinal chemistry, *Pharm. Des*. **19** (2013) 3884-3930.

[4] E. Leo, K. A. Gould, X. Su Pan, G. Capranico, M. R. Sanderson, M. Palumbo, L. M. Fisher, Novel symmetric and asymmetric DNA scission determinants for *Streptococcus pneumoniae* topoisomerase IV and gyrase are clustered at the DNA breakage site, *J. Biolog. Chem*. **280** (2005) 14252-14263.

[5] S. Bellon, J. D. Parsons, Y. Wei, K. Hayakawa, L. L. Swenson, P. S. Charifson, J. A. Lippke, R. Aldape, C. H. Gross, Crystal structures of *Escherichia coli* topoisomerase IV ParE Subunit (24 and 43 Kilodaltons): a Single residue dictates differences in novobiocin potency against topoisomerase IV and DNA gyrase, *Antimicrob. Agents Chemother*. **48** (2004) 1856-1864.

[6] S. Alt , L. A. Mitchenall , A. Maxwell and L. Heide, Inhibition of DNA gyrase and DNA topoisomerase IV of *Staphylococcus aureus* and *Escherichia coli* by aminocoumarin antibiotics, *J. Antimicrob. Chemother*. **66** (2011) 2061-2069.

[7] D. R. Reuß, P. Faßhauer, P. J. Mroch, I. Ul-Haq, B. Mo Koo, A. Pohlein, C. A. Gross, R. Daniel, S. Brantl and J. Stulke, Topoisomerase IV can functionally replace all type 1A topoisomerases in Bacillus subtilis, *Nucleic Acids Research* **47** (2019) 5231-5242.

[8] A. Gubaev, D. Weidlich and D. Klostermeier, DNA gyrase with a single catalytic tyrosine can catalyze DNA supercoiling by a nicking-closing mechanism, *Nucleic Acids Research* **44** (2016) 10354-10366.

[9] K. M. E. Roberts, C. Breuer, M. K. Wall, K. S. Shirasu and A. Maxwell, Arabidopsis thaliana GYRB3 Does Not Encode a DNA gyrase subunit, *Plos one* **5** (2010) 9899.

[10] R. P. Jaktaji, E. Mohiti, Study of mutations in the DNA gyrase gyrA Gene of *Escherichia coli*, *Iran. J. Pharm*. Res. **9** (2010) 43-48.

[11] T. Khana, K. Sankheb, V. Suvarnaa , A. Sherjea, K. Patela, B. Dravyakara, DNA gyrase inhibitors: Progress and synthesis of potent compounds as antibacterial agents, *Biomed. & Pharm* **103** (2018) 923-938.

[12] K. J. Aldred, R. J. Kerns, and N. Osherof, Mechanism of Quinolone Action and Resistance, *Biochemistry* **53** (2014) 1565-1574.

[13] M. K. Kathiravan, M. M. Khilare, K. Nikoomanesh, A.S. Chothe, K.S. Jain, Topoisomerase as target for antibacterial and anticancer drug discovery, *J. Enz. Inhib. Med. Chem.* **28** (2013) 419-435.

[14] K. J. Aldred, H. A. Schwanz, G. Li, S. A. Mcpherson, C. L. Turnbough, R. J. Kerns, N. Osheroff, Overcoming target-mediated quinolone resistance in topoisomerase IV by introducing metal-ion-independent drug-enzyme interactions, *ACS Chem. Biol*. **8** (2013) 2660-2668.

[15] T. D. M. Pham, Z. M. Ziora and M. A. T. Blaskovich, Quinolone antibiotics, *Med. Chem. Commun.* **10** (2019) 1719-1739.

[16] R. J. Fair and Y. Tor**,** Antibiotics and bacterial resistance in the 21st century, *Perspect. Medicin. Chem.* **6** (2014) 25-64.

[17] F. Collin, S. Karkare and A. Maxwell, Exploiting bacterial DNA gyrase as a drug target: current state and perspectives, *Appl. Microbiol. Biotechnol*. **92** (2011) 479-497.

[18] S. Bondock, A. E. G. Tarhoni, A. A. Fadda, Utility of cyanoacetic acid hydrazide in heterocyclic synthesis, *ARKIVOC* **9** (2006) 113-156.

[19] D. Y. Bhosale, An efficient recyclable catalytic system for the synthesis of quinoline derivatives, *Global J. of Engineering Sci. and Res.* **5** (2018) 2348-8034.

[20] D. Jabli, R. Milad, M. Abderrabba, M. L. Efrit, Synthesis, antibacterial activity and DFT calculation of naphtopyrano, furo and pyrazolo [3,2,*e*][1,2,4]-triazolo[1,5-*c*]pyrimidine derivatives, *Chemistry Africa* **2** (2019) 597-613.

[21] P. Majumdar, A. Pati, M. Patra, R. K. Behera, A. K. Behera, Acid hydrazides, potent reagents for synthesis of oxygen, nitrogen, and/or sulfur-containing heterocyclic rings, *Chem. Rev*. **114** (2014) 2942-2977.

[22] S. Urnikaite, M. Daskeviciene, R. Send, H. Wonneberger, A. Sackus, I. Bruder and V. Getautis, Organic dyes containing a hydrazone moiety as auxiliary donor for solid-state DSSC applications, *Dyes and Pigments* **114** (2015) 175-183.

[23] M. S. Khan, S. P. Siddiqui, N. Tarannum, A systematic review on the synthsis and biological activity of hydrazide derivatives, *Hygeia. J. D. Med*. **9** (2017) 61-79.

[24] R. M. Mohareb, E. E. EL-Arab, K. A. EL-Sharkawy, The reaction of cyanoacetic acid hydrazide with 2-acetylfuran: Synthesis of coumarin, pyridine, thiophene and thiazole derivatives with potential antimicrobial activities, *Sci. Pharm*. **77** (2009) 355-366.

[25] R. A. Mekheimer, M. A. Al-Sheikh, H. Y. Medrasi, G. A. A. Bahatheg, Fused quinoline heterocycles X. First synthesis of new four heterocyclic ring systems 10-amino-6,9-disubstituted-[1,2,4]triazino[4′,3′:1,5]pyrazolo[4,3-*c*]quinoline derivatives, *Synth. Comm.* **47** (2017) 1052-1064.

[26] N. Y. Gorolets, B. H. Yousefi, F. Belaj, C. O. Kappe, Rapid microwave-assisted solution phase synthesis of substituted 2-pyridone libraries, *Tetrahedron* **60** (2004) 8633-8644.

[27] H. M. Al-Matar, K. D. Khalil, A. Y. Adam, M. H. Elnagdi, Green one-pot solvent-free synthesis of pyrano[2,3-*c*]pyrazoles and pyrazolo[1,5-*a*]pyrimidines, *Molecules* **15** (2010) 6619-6629.

[28] M. Albratty, K. A. El-sharkawy, H. A. Alhazmi, Synthesis and evaluation of some new 1,3,4-oxadiazoles bearing thiophene, thiazole, coumarin, pyridine and pyridazine derivatives as antiviral agents, *Acta. Pharm*. **69** (2019) 261-276.

[29] H. Kabirifard, S. E. Mashai, N. Hamrahjoo, Synthesis of heterocyclic compounds using cyanoacetic acid hydrazide: Synthesis of pyrazolo[3,4-*b*]pyridine and pyrano[3,4-*d*]pyridazine derivatives, *Iran. J. of Org. Chem*. **5** (2013) 1111-1116.

[30] E. A. El-Rady, M. A. Khalil, 2-Cyanoacetamide in the synthesis of heterocyclic compounds: Synthesis of new polysubstituted pyrazole, pyridine and pyrimidine derivatives, *J. of the Chinese Chem. Soc*. **51** (2004) 779-784.

[31] M. A. A. Mohame, O. M. El-Hady, A. M. El-Sayed, Utility of cyanoacetic acid hydrazide in organic synthesis: Synthesis and characterization of some novel heterocycles bearing 1,3,4-oxadiazole moiety, *J. Pharm. Appl*. Chem. **3** (2017) 117-125.

[32] N. H. Metwally, M. A. Badawy, D. S. Okpy, Synthesis and anticancer activity of some new thiopyrano[2,3-*d*]thiazoles incorporating pyrazole moiety, *Chem. and Pharm. Bull*. **63** (2015) 495-503.

[33] N. H. Metwally, F. M. Abdelrazek, S. M. Eldaly, Synthesis and anticancer activity of some new heterocyclic compounds based on 1-cyanoacetyl-3,5-dimethylpyrazole, *Res. on Chem. Inter*. **42** (2016) 1071-1089.

[34] N. H Metwally, F. M Abdelrazek, S. M Eldaly, Synthesis, molecular docking, and biological evaluation of some novel bis-heterocyclic compounds based *N*,*N*′-([1,1′-biphenyl]-4,4′-diyl)bis(2-cyanoacetamide) as potential anticancer agents, *J. Heterocycl Chem*. **55** (2018) 2668-2682.

[35] N. H. Metwally, E. A. Deeb, Synthesis, anticancer assessment on human breast, liver and colon carcinoma cell lines and molecular modeling study using novel pyrazolo[4,3-*c*]pyridine derivatives, *Bioorg. Chem*. **77** (2018) 203-2014.

[36] N. H. Metwally, I. T. Radwan, W. S. El-Serwy, M. A. Mohamed, Design, synthesis, DNA assessment and molecular docking study of novel 2-(pyridin-2-ylimino)thiazolidin-4-one derivatives as potent antifungal agents, *Bioorg. Chem*. **84** (2019) 456-467.

[37] N. H. Metwally, M. S. Mohamed, E. A. Ragb, Design, synthesis, anticancer evaluation, molecular docking and cell cycle analysis of 3-methyl-4,7-

dihydropyrazolo[1,5-*a*]pyrimidine derivatives as potent histone lysine demethylases (KDM) inhibitors and apoptosis inducers, *Bioorg. Chem*. **88** (2019) 102929.

[38] N. H. Metwally, M. S. Mohamed, New imidazolone derivatives comprising a benzoate or sulfonamide moiety as anti-inflammatory and antibacterial inhibitors: Design, synthesis, selective COX-2, DHFR and molecular-modeling study, *Bioorg. Chem*. (2019) in press.

[39] S. Irshad, M. Mahmood, F. Perveen, In-Vitro anti-bacterial activities of three medicinal plants using agar well diffusion method, *Research J. of Bio*. **2** (2012) 1-8.

[40] K. M. E.Roberts, L. A. Mitchenall, M. K. Wall, J. Leroux, J. S. Mylne, A. Maxwell, DNA gyrase is the target for the quinolone drug ciprofloxacin in Arabidopsis thaliana, *J. of Bio. Chem*. **291** (2016) 3136-3144.

[41] W. Ried, E.U. Kocher, Synthesis and reactions from pyrazolo[1,5-*a*]pyrimidine, *Lieb. Ann. der Chemie* **647** (1961) 116-144.

[42] I. Wiegand, K. Hilpert, R. E. W. Hancock, *Nature Protocols* **3** (2008) 163-175.

[43] Group C. C. Chemical Computing Group and Molecular Networks Announce the Integration of CORINA into MOE 2009.

Highlights:

- A series of *N*,*N*-bis(cyanoacetyl)hydrazines and bis-coumarins designed and synthesized.
- Compound **15a** is the most toxic compound against *Escherichia coli* with activity index 111% compared to standard drug gentamicin.
- DNA gyrase and topoisomerase IV inhibition of the *N*,*N*-bis(cyanoacetyl)hydrazines and bis-coumarins was tested *in vitro.*
- Compound **15a** exhibited high potency against DNA gyrase and topoisomerase IV.

Design, green one-pot synthesis and molecular docking study of novel *N,N*-bis(cyanoacetyl)hydrazines and bis-coumarins as effective inhibitors of DNA gyrase and topoisomerase IV

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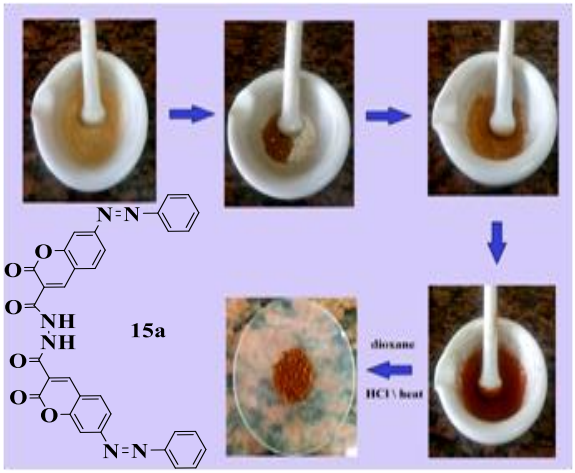

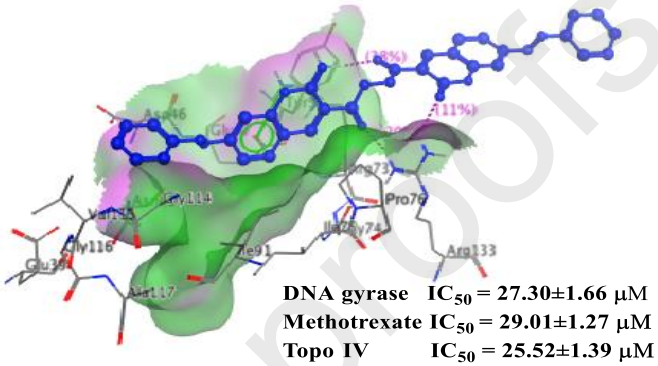

DNA gyrase IC_{50} = 27.30±1.66 μM
Methotrexate IC_{50} = 29.01±1.27 μM
Topo IV IC_{50} = 25.52±1.39 μM
Methotrexate IC_{50} = 23.55±1.71 μM

Binding pattern of compound 15a with the active site of 6ENH

Conflicts of Interest: The authors declare no conflict of interest.