Egypt. J. Chem. **Vol. 64,** No. 7 pp. 3817 - 3839 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Design, Synthesis and Molecular Docking of New Benzimidazole Derivatives of Potential Antimicrobial Activity as DNA Gyrase and Topoisomerase IV Inhibitors

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Abstract

A new series of benzimidazole derivatives **3a-3d, 4a-4c, 8a-8d, 9a,9b, 10a-10d** and **11** was synthesized and evaluated as antimicrobial agents against various gram-positive, gram-negative bacteria and fungi using vibramycin and fluconazole as positive controls for the antibacterial and antifungal activities, respectively. The examined microbial strains showed variable sensitivities against the target compounds.The examined microbial strains showed variable sensitivities against the target compounds. The minimum inhibitory concentration (MIC) was determined for the compounds showed zone of inhibition ≥ 16 mm **(4a, 4c, 8a, 10a)**. The latter derivatives were also examined as *S. aureus* DNA gyrase/topoisomerase IV inhibitors. The compounds **4a**, and **8a** represented the most promising activity for both enzymes in ATPase assay **(**IC_{50} **4a** 0.39, 0.52 and **8a** 0.66, 0.28 µM respectively) as well as the safest profile against the human normal WI38 cells upon comparing with Ciprofloxacin and Novobiocin. Compounds **8a** showed dual inhibitory effect against both targets DNA gyrase and topoisomerase IV in supercoiling and decatenation assay **(**IC_{50} 0.443 and 1.15 µM respectively). Both compounds **4a** and **8a** can be considered as lead compounds for further structural modifications to obtain more potent DNA gyrase and topoisomerase inhibitors as antibacterial agents. Molecular docking study was performed for the most promising compounds to explore the pharmacophoric moieties that governed their binding with amino acid residues of DNA gyrase and topoisomerase IV using MOE software. The results revealed a binding mode and docking scores comparable to those of a reference ligand and consistent with their DNA gyrase and topoisomerase IV inhibition activity.

Keywords: Benzimidazole; Enaminone; Schiff's Bases; Thiosemicarbazides; Antimicrobial; MIC; Gyrase; Topoisomerase IV; Cytotoxic Activity; Molecular Docking

1. Introduction

Infectious diseases severely threaten public health representing their incidence as one of the most multifaceted global health problems in past and is escalating at an unprecedented level, thereby posing an exceptional health obstacle to health care professionals [1,2]. Due to the increasing use or misuse of antibiotics as well as imprecise diagnosis, many drug-resistant pathogenic microbes have emerged in recent years [3,4]. According to the World Health Organization (WHO) reports the antimicrobial resistance (AMR) is showing an increase in alarming sound throughout the world so, it represents a serious threat to the future of human health and economy [5,6]. Microbial resistance is acquired via different mechanisms such as the overexpression of antibiotic-inactivating enzymes (e.g. extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae), mutations of the outer membrane or target enzymes as well as enhanced activity of efflux pumps [7-9]. However, the

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Receive Date: 10 May 2021, Revise Date: 24 May 2021, Accept Date: 26 May 2021
DOI: 10.21608/EJCHEM.2021.75953.3714

appearance of drug resistant strains decrease therapeutic effect of many clinical drugs or totally lose their activities and force a huge requirement to discover and develop new class of antimicrobial agents with strong activity, lower toxicity and distinct modes of action from currently clinical drugs.

Studies showed that DNA topoisomerases enzymes regulate DNA topology via cleaving and rejoining DNA strands. They introduce a crucial role to regulate the physiological actions of the genome [1–3] in addition to DNA activities such as replication, transcription [4–6], recombination [7,8], repair [9,10], chromosome decondensation and sister chromatid [11,12].

DNA gyrase and topoisomerase IV are type II topoisomerases [13,14]. DNA gyrase is composed of two GyrA and GyrB subunits, where GyrA participates in the cleavage and recombination of DNA, while the other part GyrB has ATPase activity, providing the essential energy needed for DNA cleavage and recombination. Also, topoisomerase IV has two subunits known as ParC and ParE, and it participates in decatenation of DNA and relaxation of supercoiled DNA [15,16]. Type II topoisomerase enzymes present in eukaryotic cells are unlike the prokaryotic ones, where they act as homodimer enzymes [17,18]. In addition, the binding pocket is occluded significantly in human topo II, making the interaction process of bacterial topoisomerase suppressors is less favorable. The programs of AstraZeneca investigated more than three times of selectivity for the bacterial isozymes *versus* human ones [19]. Accordingly, both DNA gyrase and topoisomerase IV are considered as significant strategic targets for the discovery of dual inhibitors of broad-spectrum antibacterial activity [20-23].

Benzimidazole is a privileged scaffold in many drugs e.g. albendazole, mebendazole, candesartan, astemizole [24,25]. It has drawn considerable attention in medicinal chemistry especially antimicrobial aspects [26,27]. It was reported that benzimidazole revealed to be specific topoisomerase inhibitors. It could bind to the minor groove of DNA and cause breakage of DNA single-strand [28]. This suggested that benzimidazole derivatives should have potential antimicrobial activity with distinct mechanism of action from the well-known antimicrobial agents, this would be helpful for these derivatives to decrease the probability of bacterial to develop resistant and accordingly enhanced their activity. Benzimidazole ring is an electron-rich nitrogen heterocycle that is beneficial for readily binding with various therapeutic targets. Accordingly benzimidazole derivatives displayed different researches revealed that benzimidazole core is considered as a bioisostere for purine base which plays a pivotal role in the bacterial cell wall, nucleic acid and protein biosynthesis. Thus, benzimidazole-based antimicrobials can interact with biopolymers as competitive inhibitors, thereby blocking the biosynthesis of the key components leading to killing or inhibiting the growth of various gram-positive, gram-negative and fungal microorganisms [29,30] (**Fig. 1**). Based on these premises, benzimidazole nucleus is considered as an indispensable good framework for the discovery of new antimicrobial active agents.

Furthermore, multiple studies showed that enaminones constitute a promising group for the development of bioactive molecules in the field of medicinal chemistry due to their lipophilicity and significant sensitivity to minor changes in their backbone structure N-C=C-C=O. In addition, they are characterized by their stability under the simulated physiological pH conditions and low toxicity [31,32]. On the other hand, sulfonamides are chemotherapeutic agents systematically used for treatment of several bacterial and fungal diseases [31,32]. Generally, sulphonamides are easy to prepare, stable and bioavailable, therefore a large number of medicinal compounds contain this functionality [33-35]. Moreover, pyridine scaffold displays an important role in designing various active antimicrobial candidates due to its specific basic structure, stability, and represents a unique class of pharmacophores due to its ability to form hydrogen bonds with different protein amino acid residues [36]. Similarly, hydrazide- hydrazones containing azomethine (–NHN=C) and thiosemicarbazide derivatives represent important classes of compounds for new antimicrobial drug development [37-39].

Molecular hybridization is a critical tool for discovery of new chemical entities and based on the aforementioned observation and in continuation of our previous studies in the antimicrobial field [40-43], the present study was focused on design and synthesis of two sets of novel benzimidazole compounds through combination of benzimidazole scaffold with different biologically active pharmacophores in a single molecular framework as shown in **(Fig.2).** These combinations may be served as a promising strategy to develop new candidates with significant antimicrobial activity with low toxicity.

The first series was based on preparation of new compounds bearing benzimidazole nucleus hybridized with different substituted aromatic and sulfonamide analogues through an enaminone linker **3a-d** and **4a-c** respectively. While the second series was obtained by molecular hybridization of benzimidazole – pyridine scaffold with various aromatic or heterocylic rings via either

oxyacetohydrazide linker **8a-c** or oxyacetothiosemicarbazide linker **9a,b**.

The newly synthesized derivatives were evaluated as antimicrobial agents against various gram-positive and negative bacterial as well as certain fungal strains. Minimum inhibitory concentrations (MIC) were investigated for the most promising compounds showing zones of inhibition ≥ 16 mm. In addition, the most promising compounds were subjected to DNA gyrase and topoisomerase IV assays to evaluate their inhibitory activities. The safety profiles of promising analogues were also assessed by examining their cytotoxicity against the human normal cells WI38. Molecular docking study was carried out to investigate their binding affinity to enzyme and to rationalize the obtained biological data.

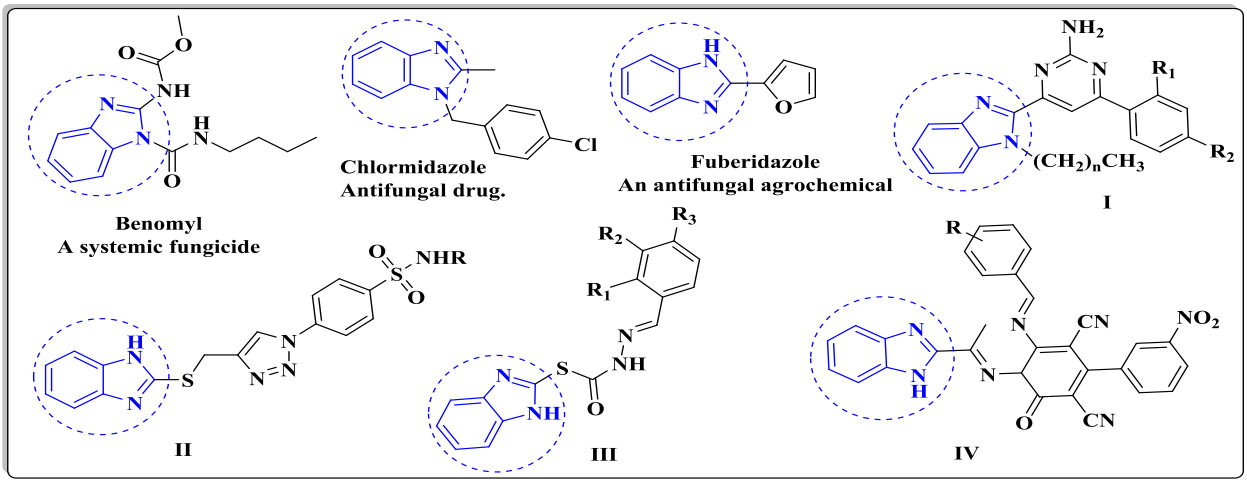


Fig.1: Different benzimidazole agents of antifungal and antibacterial activity

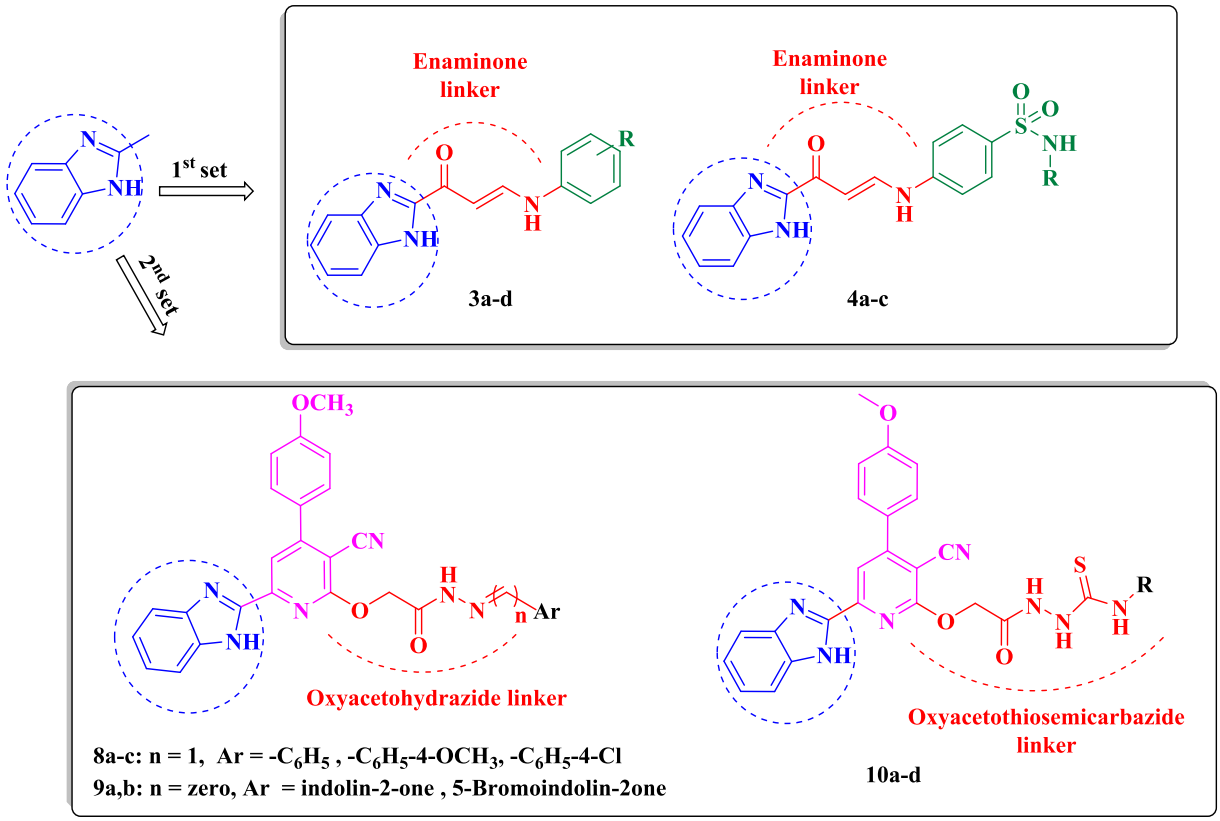


Fig. 2: Design strategy of the target benzimidazole compounds **3a-d – 10a-d**

Experimental

Chemistry

All of the chemicals and solvents were commercially available, supplied from Aldrich (Germany), Merk (Germany), Loba Chemie (India), and Fluka and used without any further purification. The melting points were uncorrected and determined in open capillary tubes on a Buchioil-heated melting point apparatus. The monitoring of the progress of all reactions and homogeneity of the synthesized compounds was carried out by thin layer chromatography (TLC) using Silica gel-precoated aluminum sheets (type 60, F 254 0.2mm thickness, Merck, Darmstadt, Germany). Visualization of compound spots on TLC and follow up were made using UV lamp at λ254 nanometer. Infrared IR spectra were recorded using potassium bromide disc technique on a Schimadzu 435 IR spectrophotometer at October 6 University. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian-Mercury 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer using DMSO-*d*6 as solvent and TMS as an internal standard at Ainshams University All chemical shifts were reported in ppm, the coupling constants (*J*) were expressed in hertz (Hz) and signals (multiplicities) were described as singlet (s), broad (br) doublet (d), triplet (t) as well as multiplet (m). Mass spectra were recorded on a DI-50 unit of Shimadzu (Tokyo, Japan) GC/MS-QP 2010 plus Spectrometer (Japan) or on single quadrpole mass Spectrometer ISQ LT (Thermo scientific) at Alazhar University. Elemental analyses (C, H, N) were performed by a Vario III CHN analyzer (Germany) at Alazhar University. All compounds were within ± 0.4% of the theoretical values. All the synthesized derivatives were chemically named based on IUPAC system.

The key intermediates 1-(1*H*-benzo[d]imidazol-2-yl)ethan-1-one (**1**) [44], *(Z/E)*1-(1*H*-benzo[*d*]imidazole-2-yl)-3-(dimethylamino)prop-2-en-1-one **(2)** [45] and 6-(1*H*-benzo[d]imidazol-2-yl)-1,2-dihydro-4-(4-methoxyphenyl)-2-oxopyridine-3-carbonitrile **(5)** [46] were prepared according to the reported method.

General procedure for synthesis of (Z/E) 3-(substitutedarylamino)-1-(1H-benzo[d]imidazol-2-yl]prop-2-en-1-one 3a-d

A mixture of the N,N dimethylenaminone derivative **2** (0.43 g, 2 mmole) and the respective primary aromatic amines namely; *p*-hydroxy, *p*-methoxy, *m*-chloro, *m*-bromoaniline (20 mmole) in acetic acid (10 mL) was refluxed for 5 h. After cooling the formed precipitate was filtered, washed with water, dried and recrystallized from ethanol to give the title compounds **3a–d** respectively.

(Z/E) 1-[1H-benzo[d]imidazol-2-yl)-3-(4-hydroxy phenyl)amino]prop-2-en-1-one (3a)

Yield (74%); orange powder; m.p. 230-233 °C; IR: (cm^{-1}): 3250 (OH), 3155, 3122 (2NH), 1640 (C=O); ^{1}H NMR (δ ppm): 6.43, 6.80 (2d, 1H, *J*= 7.6, 8.8Hz, –COCH= *Z / E*), 7.05–7.94 (m, 9H, Ar-H, =CH–N), 9.32, 9.45 (2s,1H, OH, exchangeable, *E/Z*), 10.29, 11.98 (2d, 1H, *J*=13.2 and 12.8 Hz, NH- enaminone, exchangeable with D_2O, *E/Z*), 12.99 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 93.13, 112.93, 116.65, 117.95, 118.78, 120.82, 122.75, 132.19, 135.39, 143.82, 148.14, 150.63, 151.32, 154.93, (Aromatic-C and CH=CH), 178.97, 180.46 (C=O, *Z/E*); MS (*m/z*, %): $M^{+\cdot}$, 279 (38.71%), 132.69 (100%); Anal. Calcd For $C_{16}H_{13}N_3O_2$ (279.30): % C, 68.81; H, 4.69; N, 15.05. Found: % C, 68.97; H, 4.81; N, 15.23.

(Z/E) 1-[1H-benzo[d]imidazol-2-yl)-3-(4-methoxy phenyl)amino]prop-2-en-1-one (3b)

Yield (80%); dark brown powder; m.p. 250-253 °C; IR (cm^{-1}): 3257, 3122 (2NH), 1640 (C=O); ^{1}H NMR (δ ppm): 3.77 (s, 3H, OCH_3), 6.40, 6.99 (2d, 1H, *J*= 7.6, 8.8 Hz, –COCH = *Z/E*), 7.17-8.32 (m, 9H, Ar-H, =CH-N), 10.33, 11.95 (2d, *J*= 8.0, 12.8 Hz, 1H, NH-enaminone, exchangeable with D_2O, *Z/E*), 13.03 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.80 (OCH_3), 93.30, 113.05, 115.43, 118.54, 120.69, 122.95, 123.14, 124.51, 133.60, 134.95, 135.08, 143.65, 147.99, 151.19, 156.44 (aromatic-C and CH=CH), 180.34, 180.55 (C=O, *Z/E*); MS (*m/z*, %): $M^{+\cdot}$ 293 (68.73), 149.62 (100%); Anal. Calcd For $C_{17}H_{15}N_3O_2$ (293.33): % C, 69.61; H, 5.15; N, 14.33. Found: % C, 69.88; H, 5.39; N, 14.59.

(Z/E) 1-(1H-benzo[d]imidazol-2-yl)-3-[(3-chloro phenyl)amino]prop-2-en-1-one (3c)

Yield (75%); yellow powder; m.p.234–236 °C; IR (cm^{-1}): 3362, 3251 (2NH), 1641 (C=O); ^{1}HNMR (δ ppm): 6.55, 6.97 (2d, 1H, *J* = 8.0, 13.2 Hz, -COCH =, *Z/E*), 7.08–8.44 (m, 9H, Ar-H, =CH-N), 10.47, 11.73 (2d, 1H, *J* = 12.4, 12.4 Hz, NH-enaminone, exchangeable with D_2O, *Z/E*), 13.15 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 95.12, 112.85, 115.75, 121.24, 122.88, 123.91, 124.83, 131.65, 134.95, 141.99, 142.89, 144.56, 146.52, 149.94, 150.56 (aromatic-C and CH=CH), 179.68, 181.54 (C=O, *Z/E*); MS (*m/z*, %): $(M+2)^{+\cdot}$ 299 (15.51), 181.5 (100). Anal. Calcd For $C_{16}H_{12}ClN_3O$ (297.74): % C, 64.54; H, 4.06; N, 14.11. Found: % C, 64.32; H, 4.21; N, 14.29.

(Z/E) 1-[(1H-benzo[d]imidazol-2-yl)-3-(3-bromo phenyl)amino]prop-2-en-1-one (3d)

Yield (65%); brown powder; m.p. 254–256°C; IR (cm^{-1}): 3362, 3251 (2NH), 1641 (C=O). ^{1}HNMR (δ

ppm): 6.54, 6.88 (2d, 1H, *J*= 8.00, 16.00 Hz, –COCH= *Z/ E*), 7.20–8.43 (m, 9H, Ar–H, =CH-N) 10.45, 11.72 (2d, 1H, *J* =12.0, 8.0 Hz, NH-enaminone, exchangeable with D_2O, *E/Z*), 13.12 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 95.12, 113.00, 115.81, 121.27, 122.71, 123.99, 124.83, 131.65, 134.72, 142.89, 144.68, 145.30 (aromatic-C and CH=CH), 179.69, 184.04 (C=O, *Z/E*); MS (m/z, %): $(M+1)^{+\cdot}$ 343 (28.31), 338.82 (100%); Anal. Calcd for $C_{16}H_{12}BrN_3O$ (342.20): % C, 56.16; H, 3.53; N, 12.28. Found: % C, 56.38; H, 3.70; N, 12.28.

General procedure for synthesis of 4-[3-(1H-benzo[d]imidazol-2-yl)-3-oxoprop-1-en-1-yl)amino] substituted benzenesulfonamides 4a-c

A mixture of the enaminone derivative **2** (0.43 g, 2 mmole) and the appropriate sulpha drugs namely; sulfacetamide, sulfamethoxazole, sulfamethazine (2 mmole) in acetic acid (10 mL) was refluxed for 2h. After reaction completion, the formed precipitate was filtered, washed several times with water, dried and crystallized from ethanol to give the corresponding compounds **4a-c** respectively.

(Z/E) N-[(4- (3-(1H-benzo[d]imidazol-2-yl)-3-oxoprop-1-en-1-yl)amino)phenyl) sulfonyl] acetamide (4a)

Yield (80%); yellowish powder; m.p. 274–276 °C; IR (cm^{-1}): 3319, 3257 (3NH), 1650 (C=O), 1279, 1095 (SO_2); ^{1}HNMR (δ ppm): 1.92 (s, 3H, $COCH_3$), 6.68, 7.01 (2d, 1H, *J* = 8.0, 12.8 Hz, –COCH=, *Z/E*), 7.32–8.46 (m, 9H, Ar-H, =CH–N, *Z/E*), 10.70, 11.81 (2d, 1H, *J* = 12.8, 12.4 Hz, NH-enaminone, exchangeable with D_2O, *Z/E*), 11.98 (1s, 1H, SO_2NH, exchangeable with D_2O), 13.17 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 22.98 ($CO\underline{CH_3}$), 96.37, 101.36, 113.05, 115.76, 116.64, 120.99, 125.11, 129.79, 132.24, 133.69, 134.58, 143.83, 144.50, 145.38, 150.39, 168.88 (aromatic-C and CH=CH), 169.10 ($\underline{CO}CH_3$), 180.14, 181.81 (C=O, *Z/E*); MS (m/z, %): 384 ($M^{+\cdot}$, 41), 333 (100%); Anal. Calcd for: $C_{18}H_{16}N_4O_4S$ (384.41): C, 56.24; H, 4.20; N, 14.58; S, 8.34; found: C, 56.13; H, 4.51; N, 14.32; S, 8.50.

(Z/E)4-[(3-(1H-benzo[d]imidazol-2-yl)-3-oxoprop-1-en-1-yl)amino)-N-(5-methylisoxazol-3-yl]benzene sulfonamide (4b)

Yield (60%); buff powder; m.p. 278–280 °C; IR: (cm^{-1}): 3303, 3171 (3NH), 1636 (C=O), 1279, 1157 (SO_2); ^{1}H NMR (δ ppm): 1.94 (s, 3H, CH_3), 6.64, 7.01 (2d, 1H, *J* =21.6 and 16 Hz, –COCH=, *Z/ E*), 7.29–8.52 (m, 10H, Ar-H, isoxazole-H_4, =CH–N, *Z/E*), 10.69 and 11.82 (2d, 1H, *J* = 17.6, 11.6 Hz, enaminone-NH, exchangeable with D_2O, *E/Z*), 11.97 (bs, 1H, SO_2NH, exchangeable with D_2O), 13.19 (s, 1H, NH-benzimidazole, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 23.0 (CH_3), 96.43, 101.48, 116.63, 125.19, 130.04, 132.24, 135.19, 143.55, 144.99, 145.63, 146.18, 149.80, 150.56, 169.25 (aromatic-C and CH=CH), 180.14, 182.09 (C=O, *Z/E*) ; MS (m/z, %): 424.19 ($M^{+\cdot}$, 31.60), 245.91 (100%); Anal. Calcd for: $C_{20}H_{17}N_5O_4S$ (423.45): C, 56.73; H, 4.05; N, 16.54; found: C, 57.01; H, 4.26; N, 16.68.

(Z/E)4-[(3-(1H-benzo[d]imidazol-2-yl)-3-oxoprop-1-en-1-yl)amino)-N-(4,6-dimethylpyrimidin-2-yl] benzenesulfonamide (4c)

Yield (75%); yellow powder; m.p. 282–284°C, IR: (cm^{-1}): 3303, 3171 (3NH),1640 (C=O), 1279, 1157 (SO_2); ^{1}H NMR (ppm): 2.23 (s, 6H, $2CH_3$), 6.59, 6.97 (2d, 1H, *J* = 8.0, 13.2 Hz, –COCH=, *Z/E*), 6.77 (s, 1H, pyrimidine-H_5), 7.25–8.46 (m, 9H, Ar-H, =CH–N), 10.62, 11.79 (2d, 1H, *J* = 12.8, 12.4 Hz, NH-enaminone, exchangeable with D_2O, *Z/E*), 11.42 (s, 1H, SO_2NH, exchangeable with D_2O), 13.15 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 23.37, 26.31 ($2CH_3$), 100.77, 104,79, 113.61, 113.85, 116.20, 123.33, 123.60, 125.11, 30.47, 131.02, 134.87, 143.46, 144.06, 150.59, 154.79, 156.30, 157.02, 167.31 (aromatic-C and CH=CH), 176.31, 180.43 (C=O, *Z/E*); MS (m/z, %): 448 ($M^{+\cdot}$, 79); Anal. Calcd for: $C_{22}H_{20}N_6O_3S$ (448.50): C, 58.92; H, 4.49; N, 18.74; found: C, 59.04; H, 4.57; N, 18.96.

Ethyl 2-((6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy)acetate (6)

A mixture of the pyridone compound **5** (0.40 g, 1mmole), ethyl bromoacetate (0.12 mL, 1 mmol) and anhydrous potassium carbonate (0.21 g, 15 mmole) in dry acetone (20 mL) was refluxed for 6h with stirring. The reaction mixture was cooled and poured onto ice/cold water. The formed precipitate was filtered, dried and recrystallized from methanol to give the corresponding derivative **6**.

Yield (80%); light yellow powder; m.p. 220–222°C; IR (cm^{-1}): 3303 (NH), 2220 (CN), 1666 (C=O); ^{1}HNMR (δ ppm): 1.20 (t, 3H, CH_3), 3.83 (s, 3H, OCH_3), 4.20 (q, 2H,CH_2), 5.20, (S, 2H, OCH_2), 7.16–8.22 (m, 9H, Ar-H, pyridine-H_5), 12.70 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C NMR (δ ppm): 14.40 (CH_3), 55.94 (OCH_3), 61.64 ($2CH_2$), 94.15, 112.70, 114.42, 115.54, 117.87, 120.36, 123.91, 125.04, 127.45, 130.62, 137.42, 142.17, 147.61, 148.70, 149.11, 156.36, 161.56, 163.64, 164.06 (aromatic-C and CN), 168.26 (C=O); MS (m/z,%): 428.25 ($M^{+\cdot}$, 16.22), 77.71 (100%); Anal. Calcd. for $C_{24}H_{20}N_4O_4$ (428.45): C, 67.28; H, 4.71; N, 13.08; found: C, 67.04; H, 4.89; N, 13.26.

2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy]aceto hydraziде (7)

A mixture of the ester **6** (1.0 g, 2 mmole) and hydrazine hydrate 98% (0.2 mL, 4 mmole) in absolute ethanol (30 mL) was refluxed with stirring for 6h. The formed precipitate was filtered and recrystallized from acetic acid to give the hydrazide derivative **7**.

Yield (60%); yellow powder, m.p. 252–254°C; IR (cm^{-1}): 3413, 3254 (NH_2, 2NH), 2216 (CN), 1669 (C=O); ^{1}HNMR (δ ppm): 3.87 (s, 3H, OCH_3), 4.55 (bs, 2H, NH_2, exchangeable with D_2O), 5.20 (S, 2H, OCH_2), 7.13 - 8.68 (m, 9H, Ar-H, pyridine-H_5), 9.37 (s, 1H, NH-hydrazide, exchangeable with D_2O), 13.10 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.53 (OCH_3), 56.14 (OCH_2), 94.15, 112.70, 115.44, 117.27, 121.36, 123.91, 125.84, 127.42, 130.62, 137.28, 141.17, 147.61, 148.70, 149.01, 156.01, 160.04, 161.73, 164.06 (aromatic-C and CN), 168.77 (C=O); MS (m/z, %): 414 ($M^{+\cdot}$, 43.6), (115.42, 100%); Anal. Calcd. for $C_{22}H_{18}N_6O_3$ (414. 43): Calcd. C, 63.76; H, 4.38; N, 20.22; found: C, 63.89; H, 4.54; N, 19.99.

General procedure for synthesis of 2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxy phenyl)pyridin-2-yl)oxy)-N'-(substituted benzylidene] hydrazones derivatives 8a-c and 2-((6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxy phenyl)pyridin-2-yl)oxy)-N'-(5-substituted-2-oxoindolin-3-ylidene) acetohydrazide 9a,b

A mixture of the acetohydrazide derivative **7** (0.4 gm, 1 mmole) and the appropriate carbonyl compound aromatic aldehydes namely; benzaldehyde, *p*-methoxybenzaldehyde, *p*-chlorobenzaldehyde or isatin and/or 5-bromo isatin) (1 mmole) in EtOH (10 mL) in presence of few drops of glacial acetic acid was refluxed for 6-8h. After reaction completion, the reaction mixture solution was cooled and the obtained precipitate was filtered and recrystallized from dichloromethane to achieve the hydrazone derivatives **8a-c** and **9a,b** respectively.

2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy]-N'-benzylidene acetohydrazide (8a)

Yield (84%); white solid; m.p. > 300°C; IR (cm^{-1}): 3303, 3171 (2NH), 2216 (CN), 1640 (C=O); ^{1}HNMR (δ ppm): 3.82 (s, 3H, OCH_3), 5.39, (S, 2H, OCH_2), 6.99 - 8.08 (m, 14H, Ar-H, pyridine-H_5), 8.32 (s, 1H, -N=CH), 11.65, 11.75 (2s, 1H, =N-NH, exchangeable with D_2O), 13.16 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.42 (OCH_3), 64.55 (OCH_2), 94.10, 104.28, 115.03, 120.23, 122.72, 123.03, 127.43, 127.69, 130.71, 134.57, 135.12, 140.34, 142.01, 144.25, 144.81, 148.91, 149.47, 156.60, 158.59, 161.58, 165.48, 169.02, 169.58 (Ar-C, CH=N and CN), 175.35 (C=O); MS (m/z,%): 502 ($M^{+\cdot}$, 19.76), 439.08 (100%); Anal. Calcd. for $C_{29}H_{22}N_6O_3$ (502.53): Calcd. C, 69.31; H, 4.41; N, 16.72; found: C, 69.59; H, 4.65; N, 16.51.

2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy)-N'-(4-methoxy benzylidene] acetohydrazide (8b)

Yield (75%); white solid; m.p. 296–298°C; IR (cm^{-1}): 3303, 3171 (2NH), 2218 (CN), 1643 (C=O); ^{1}HNMR (δ ppm): 3.82, 3.89 (2s, 6H, $2OCH_3$), 5.41 (s, 2H, OCH_2), 6.99 - 8.08 (m, 13H, Ar-H, pyridine-H_5), 8.27 (s, 1H, -N=CH), 11.59, 12.00 (2s, 1H, NH, exchangeable with D_2O), 13.05 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.42, 55.85 ($2OCH_3$), 61.10 (OCH_2), 92.17, 114.10, 115.41, 120.92, 122.65, 123.39, 124.92, 128.70, 129.01, 130.62, 130.91, 134.58, 135.50, 136.64, 139.64, 142.37, 144.81, 148.98, 149.07, 150.59, 158.59, 161.39, 164.61 (Ar-C, CH=N and CN), 177.27 (C=O); MS (m/z,%): 532 ($M^{+\cdot}$, 19.76), 439.08 (100%); Anal. Calcd. for $C_{30}H_{24}N_6O_4$ (532.56): Calcd. C, 67.66; H, 4.54; N, 15.78; found: C, 67.31; H, 4.27; N, 15.54.

2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy)-N'-(4-chloro benzylidene] acetohydrazide (8c)

Yield (82%); white solid; m.p. > 300; IR (cm^{-1}): 3303, 3171 (2NH), 2220 (CN), 1645 (C=O); ^{1}HNMR (δ ppm): 3.89 (s, 3H, OCH_3), 5.43 (s, 2H, CH_2), 7.18-8.33 (m, 14H, Ar-H, pyridine-H_5, -N=CH), 11.69 (s,1H, NH, exchangeable with D_2O), 13.05 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.72 (OCH_3), 60.30 (OCH_2), 94.14, 115.10, 115.71, 123.54, 125.56, 127.01, 128.45, 129.11, 130.01, 130.70, 133.47, 135.02, 137.46, 140.21, 144.25, 145.67, 150.90, 154.43, 155.51, 156.64, 158.03, 169.10, 172.31 (Ar-C, CH=N and CN), 180.10 (C=O); MS (m/z,%): 537.72 ($M^{+\cdot}$ + 1, 30.17), 50.34 (100%); Anal. Calcd. for $C_{29}H_{21}ClN_6O_3$ (536.98): Calcd. C, 64.87; H, 3.94; N, 15.65; found: C; 65.03; H, 4.16; N, 15.31.

2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy]-N'-(2-oxoindolin-3-ylidene)acetohydrazide(9a)

Yield (75%); yellow solid; m.p. > 300; IR (cm^{-1}): 3303, 3245, 3171 (3NH), 2217 (CN), 1668, 1640 (2C=O); ^{1}HNMR (δ ppm): 3.89 (s, 3H, OCH_3), 6.38 (s, 2H, CH_2), 7.05-8.18 (m, 13H, Ar-H, pyridine-H_5), 10.76, 11.03 (s, 2H, 2NH, exchangeable with D_2O), 13.12 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.99 (OCH_3), 60.26 (OCH_2), 94.11, 114.11, 115.71, 122.58, 125.90, 126.21, 127.32, 128.45, 129.11, 130.01, 131.25, 133.47, 134.12, 136.29, 141.21, 143.05, 145.67, 148.8, 150.90, 154.69, 157.61, 158.33, 165.12 (Ar-C

and CN), 175.48, 180.10 (2C=O); MS (m/z,%): 544 ($M^{+\cdot}$+1, 20.17), 50.34 (100%); Anal. Calcd. for $C_{30}H_{21}N_7O_4$ (543.54): Calcd. C, 66.29; H, 3.89; N, 18.04; found: C, 66.58; H, 4.10; N, 18.32.

2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy]-N'-(5-bromo-2-oxoindolin-3-ylidene) acetohydrazide (9b)

Yield (69%); brown solid; m.p. > 300; IR (cm^{-1}): 3355, 3250, 3185 (3NH), 2217 (CN), 1670, 1640 (2C=O); ^{1}HNMR (δ ppm): 3.87 (s, 3H, OCH_3), 6.38 (s, 2H, CH_2), 7.18-8.01 (m, 12H, Ar-H, pyridine-H_5), 10.76, 11.41 (2s, 2H, 2NH, exchangeable with D_2O), 13.10 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.72 (OCH_3), 60.30 (OCH_2), 99.88, 105.35, 110.50, 114.11, 115.36, 120.11, 120.31, 126.91, 130.77, 131.25, 133.77, 134.12, 138.89, 141.81, 140.95, 145.27, 148.8, 152.50, 152.99, 153.45, 161.36 167.53, 168.53 (Ar-C and CN), 175.48, 180.10 (2C=O); MS (m/z,%): 623 ($M^{+\cdot}$+1, 10.17), 50.34 (100%); Anal. Calcd. for $C_{30}H_{20}BrN_7O_4$(622.44): Calcd. C, 57.89; H, 3.24; N, 15.75; Found: C, 58.12; H, 3.50; N, 15.99.

General procedure for synthesis of 2-[2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxy phenyl)pyridin-2-yl)oxy]acetyl]-N-substituted-hydrazine-1-carbothioamides 10a-d

A mixture of the acetohydrazide derivative **7** (0.4 g, 1 mmole) and the appropriate isothiocyante derivatives namely; allylisothiocyanate, phenyl isothiocyanate, 3-nitrophenylisothiocyanate, 3-bromophenylisothiocyanate (1 mmole) in DMF (10 mL) was refluxed with stirring for 8-10h. The formed precipitate was filtered, washed with petroleum ether, and recrystallized from dichloromethane to give the corresponding thiosemicarbazides **10a-d**.

2-[2-[(6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy]acetyl]-N-allyl hydrazine-1-carbothioamide(10a)

Yield (62%); light yellow powder; m.p. 233-235°C; IR (cm^{-1}): 3418, 3350, 3145 (4NH), 2218 (CN), 1645 (C=O), 1351 (C=S); ^{1}HNMR (δ ppm): 3.87 (s, 3H, OCH_3), 4.19 (d, 2H, -HN-C<u>H</u>$_2$), 5.07, 5.10 (2m, 2H, =C<u>H</u>$_2$), 5.36, (s, 2H, OCH_2), 5.83 (m, 1H, -C<u>H</u>=CH_2), 7.17-8.20 (m, 9H, Ar-H, 1H, pyridine-H_5), 8.72, 9.21, 9.43 (3s, 3H, 3NH, exchangeable with D_2O), 13.10 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 56.05 (OCH_3), 58.91 (OCH_2), 114.50, 115.03, 115.70, 117.38, 123.11, 124.61, 128.06, 128.49, 129.61, 129.98, 130.55, 130.71, 133.60, 148.70, 149.07, 156.37, 160.54, 161.86 (Ar-C and CN), 169.53 (C=O), 180.39 (C=S); MS (m/z,%): 513 ($M^{+\cdot}$, 27.53), 249.93 (100%); Anal. Calcd. for $C_{26}H_{23}N_7O_3S$ (513.58): C, 60.81; H, 4.51; N, 19.09; found: C, 61.04; H, 4.67; N, 19.36.

2-[2-((6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy)acetyl]-N-phenyl hydrazine-1-carbothioamide (10b)

Yield (75%); light yellow powder; m.p. 196-198°C; IR (cm^{-1}): 3418, 3350, 3199 (4NH), 2217 (CN), 1699 (C=O), 1329 (C=S); ^{1}HNMR (δ ppm): 3.87 (s, 3H, OCH_3), 5.81 (s, 2H, -OCH_2), 7.17-7.96 (m, 16H, Ar-H, pyridine-H_5, 2NH, exchangeable with D_2O), 13.10, 14.17 (2s, 2H, 2NH, exchangeable with D_2O), ; ^{13}C-NMR (δ ppm): 55.81 (OCH_3), 58.95 (OCH_2), 114.50, 115.03, 115.70, 117.38, 120.53, 123.11, 124.61, 128.06, 128.24, 129.61, 129.98, 130.55, 130.71, 133.60, 134.77, 136.02, 148.70, 149.07, 156.37, 160.55, 161.86 (Ar-C and CN), 169.53 (C=O), 180.39 (C=S); MS (*m/z*,%): 549 ($M^{+\cdot}$, 12.40), 427.28 (100%); Anal. Calcd. for $C_{29}H_{23}N_7O_3S$ (549.61): Calcd. 63.38; H, 4.22; N, 17.84; found: 63.14; H, 4.53; N, 17.99.

2-[2-((6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl) pyridin-2-yl) oxy) acetyl]-N-(3-nitrophenyl)hydrazine-1-carbothioamide (10c)

Yield (62%); light yellow powder; m.p. 233-235°C; IR (cm^{-1}): 3420, 3250, 3145 3388 (4NH), 2222 (C≡N), 1686 (C=O), 1329 (C=S); ^{1}HNMR (δ ppm): 3.87 (s, 3H, OCH_3), 5.81 (s, 2H, OCH_2), 7.17-7.96 (m, 13H, Ar-H, pyridine-H_5), 9.82, 9.89, 10.36 (3s, 3H, 3NH, exchangeable with D_2O) 12.92 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.81 (OCH_3), 58.95 (OCH_2), 95.10, 109.57, 114.87, 115.45, 115.90, 117.55, 120.53, 123.50, 124.77, 126.48, 130.14, 130.74, 132.08, 134.77, 136.02, 140.90, 156.21, 159.57, 160.55, 161.50 (Ar-C and CN), 165.82 (C=O), 182.29 (C=S); MS (m/z,%): 594 ($M^{+\cdot}$, 12.40), 352 (100%); Anal. Calcd. for $C_{29}H_{22}N_8O_5S$ (594.61): Calcd. C, 58.58; H, 3.73; N, 18.85; found: C, 58.23; H, 3.51; N, 18.54.

2-[2-((6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)oxy)acetyl)-N-(3-bromophenyl) hydrazine-1-carbothioamide (10d)

Yield (79%); yellow powder; m.p. 260-263°C; IR (cm^{-1}): 3420, 3388, 3145 (4NH), 2222 (C≡N), 1686 (C=O), 1329 (C=S); ^{1}HNMR (DMSO-*d6*, δ ppm): 3.87 (s, 3H, OCH_3), 3.87 (s, 2H, -OCH_2), 7.18-8.20 (m, 13H, Ar-H, pyridine-H_5), 10.37, 10.44, 11.60 (3s, 3H, 3NH, exchangeable with D_2O), 12.39 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.77 (OCH_3), 55.93 (OCH_2), 91.39, 112.90, 114.95, 117.79, 120.65, 121.73, 125.12, 126.86, 130.59, 131.51, 135.01, 138.64, 140.21, 143.47, 143.79, 144.52, 146.08, 147.12, 149.97, 155.12 (Ar-C and CN), 169.39 (C=O), 188.20 (C=S); MS (m/z,%): 627.98 ($M^{+\cdot}$, 12.40), 427.28 (100%); Anal. Calcd. $C_{29}H_{22}BrN_7O_3S$ (628.51): Calcd. C, 55.42; H, 3.53; N, 15.60. Found: C, 55.70; H, 3.69; N, 15.81.

Synthesis of 2-((6-(1H-benzo[d]imidazol-2-yl)-3-cyano-4-(4-methoxyphenyl) pyridin-2-yl)oxy)-N'-formylacetohydrazide (11)

A mixture of the acetohydrazide derivative **7** (0.8 g, 2 mmole) and formic acid (10 mL) was refluxed with stirring for 2h. The formed precipitate was filtered, washed with petroleum ether, dried and recrystallized from ethanol to give the title compound **11.**

Yield (82%); white solid; m.p. 196–198°C; IR (cm^{-1}): 3425, 3356, 3145 (3NH), 2222 (C≡N), 1686, 1670 (2C=O); ^{1}HNMR (δ ppm): 3.88 (s, 3H, OCH_3), 5.12 (s, 2H, OCH_2), 7.18-7.98 (m, 8H, Ar-H), 8.01 (s, 1H, pyridine-H_5), 8.22 (s, 1H, CHO), 10.01, 10.34 (s, 2NH, exchangeable with D_2O), 13.01 (s, 1H, benzimidazole-NH, exchangeable with D_2O); ^{13}C-NMR (δ ppm): 55.91 (OCH_3), 64.36 (OCH_2), 94.36, 113.05, 143.55, 143.85, 144.76, 145.70, 149.93, 150.55 (Aromatic-C and CN), 169.00, 181.98 (2C=O); MS (*m/z*,%): 442.44 ($M^{+\cdot}$, 12.40), 427.28 (100%); Anal. Calcd. for $C_{23}H_{18}N_6O_4$ (442.43): Calcd. C, 62.44; H, 4.10; N, 19.00; Found: C, 62.28; H, 4.33; N, 19.21.

Antimicrobial Activity

Antimicrobial Activity Using Agar well - Diffusion Method

The chemical compounds were individually tested against a panel of gram positive and gram-negative bacterial pathogens and fungi. The antimicrobial tests were carried out by the agar well diffusion method [47,48] using 100µL of suspension containing 1 x10^8 CFU/mL of pathological tested bacteria and 1 x10^6 CFU/ml of yeast spread on nutrient agar (NA), and Sabourand dextrose agar (SDA) respectively. After the media had cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 100 µL of the tested compound solution prepared by dissolving10 mg of the chemical compound in one mL of Dimethyl Sulfoxide (DMSO). The inculcated plates were then incubated for 24 h at 37 °C for bacteria and yeast, 48h at 28°C for fungi. Negative controls were prepared using DMSO employed for dissolving the tested compounds. Vibramycin and ciprofloxacin (10 mg/mL) and Ketoconazole (10 mg/mL) were used as standard drugs for antibacterial and antifungal activity respectively. After incubation time, the antimicrobial activity was evaluated by measuring the zones of inhibition against the test organisms and compared with that of the standard drugs. The antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm). The experiments were carried out in triplicates and the average zones of inhibition were calculated.

Minimum Inhibitory Concentration (MIC) Measurement

The bacterial activity of the active compounds (having inhibition zones (IZ) ≥ 16 mm) was then evaluated using the two-fold serial dilution technique [49,50]. Two-fold serial dilutions of the tested compounds solutions were prepared using the proper nutrient broth. The final concentrations of the solutions were 1000, 500, 250, and 125 µg/mL. The tubes were then inoculated with the test organisms, grown in their suitable broth at 37°C for 24 hours for the tested microorganisms (1×10^8 CFU/mL for bacteria and 1 x10^6 CFU/mL of yeast), each 5 mL received 0.1 mL of the above inoculum and incubated at 37°C for 24 h. The lowest concentration showing no growth was taken as the minimum inhibitory concentration (MIC).

Enzyme assessment of DNA gyrase and topoisomerase IV

DNA gyrase supercoiling assay

The supercoiling assay of DNA gyrase has been carried out at the confirmatory diagnostic R&D sector (*Vacsera-Egypt*) using S.aureus DNA Gyrase commercially available assay kit (Inspiralis) for the determination of IC_{50} values. Details of the materials; *S. aureus* Gyrase Assay Buffer, Dilution Buffer, Enzyme (Store at -80^{0}C) and Plasmid**,** Serial dilutions of each compound were prepared using DMSO as solvent. IC_{50} values were determined at a final concentration of 100, 10, 1, and 0.1 µg/ml. Novobiocin and ciprofloxacin were used as reference standards [51,52].

Topoisomerase IV decatenation assay

Commercially available assay kit (Inspiralis) was used for the determination of Topo IV decatenation IC_{50} values. Details of the materials; *S. aureus* Gyrase Topo IV Assay Buffer, Dilution Buffer, Enzyme (Store at -80^{0}C) and substrate: kDNA (supplied at 100 ng/µL, Store at -20^{0}C or below)**.** Serial dilutions of each compound were prepared using DMSO as solvent. IC_{50} values were determined at a final concentration of 100, 10, 1, and 0.1 µg/ml. Novobiocin and ciprofloxacin were used as reference standards. Novobiocin and ciprofloxacin were used as reference standards [53].

DNA gyrase and topoisomerase IV ATPase assays

Commercially available S.aureus Gyrase ATPase Assay Kit (inspiralis) and S. aureus Topoisomerase IV ATPase Assay Kit (inspiralis) were used for determination of IC50 values. Briefly, Assay Mix was set up of Assay Buffer (20 µL of 5× buffer per assay), linear pBR322 (3 µL per assay), 1 µL PEP, 1.5 µL PK/LDH, 2 µL NADH and 45.8 µL water. 73.3 µL of Assay Mix was added into the wells of the microtitre plate. 10 µL of inhibitors was added to the test wells. 10 µL of enzyme (S. aureus Gyrase/S.

aureus Topoisomerase IV) was added. Plate was put in plate reader and absorbance at OD 340 nm was monitored for 10 min at 25 °C. The plate reader was stopped. 6.7 µL of ATP was added to each well. This starts the reaction. The plate was returned to plate reader and absorbance was monitored at OD 340 nm for up to 60 min at 25 °C. Novobiocin and ciprofloxacin were used as reference standards [54].

***In vitro* cytotoxicity assay**

The cytotoxic effect of test samples using WI38 cells was evaluated by MTT assay [55-57]. Commercially available kit for in vitro toxicology MTT based assay, Sigma was used. Briefly, WI38 cells were grown as monolayer culture in DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone), penicillin (100 µg/mL) and streptomycin (100 µg/mL) and maintained under an atmosphere of 5% CO_2 at 37 °C. Control cells were incubated for 48 h at 37 °C in culture medium. Cells were rinsed with PBS and harvested by trypsinization and were plated in 96 well plates and incubated under 5% CO_2 at 37 °C overnight. Different concentrations of test samples were used for the treatment of cells. All the test samples were removed after incubation for 48 h at 37 °C and 100 µL of MTT (5 mg/mL) was added and again incubated for 4 h at 37 °C and kept under dark condition. Then, 100 µL of MTT solubilizing solution was added and incubated for 1 h at 37 °C. The absorbance was read at λ 590 nm using microtitre plate reader and cell viability was calculated. Chemicals and reagents were from Sigma, or Invitrogen.

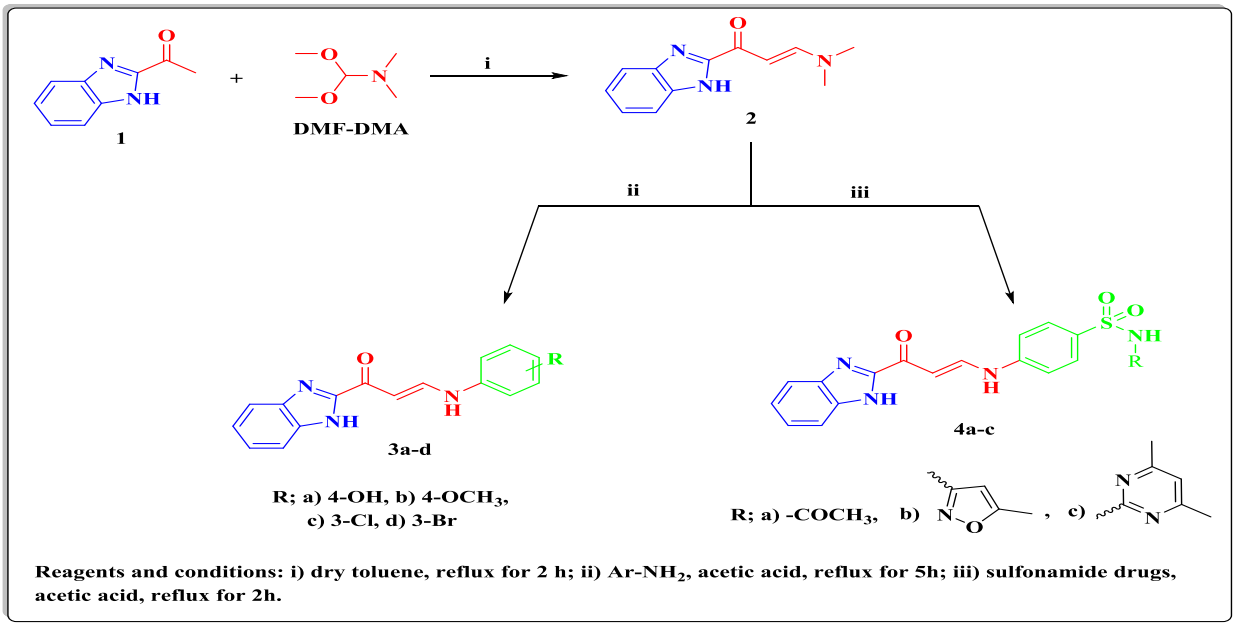
Results and discussion

Chemistry

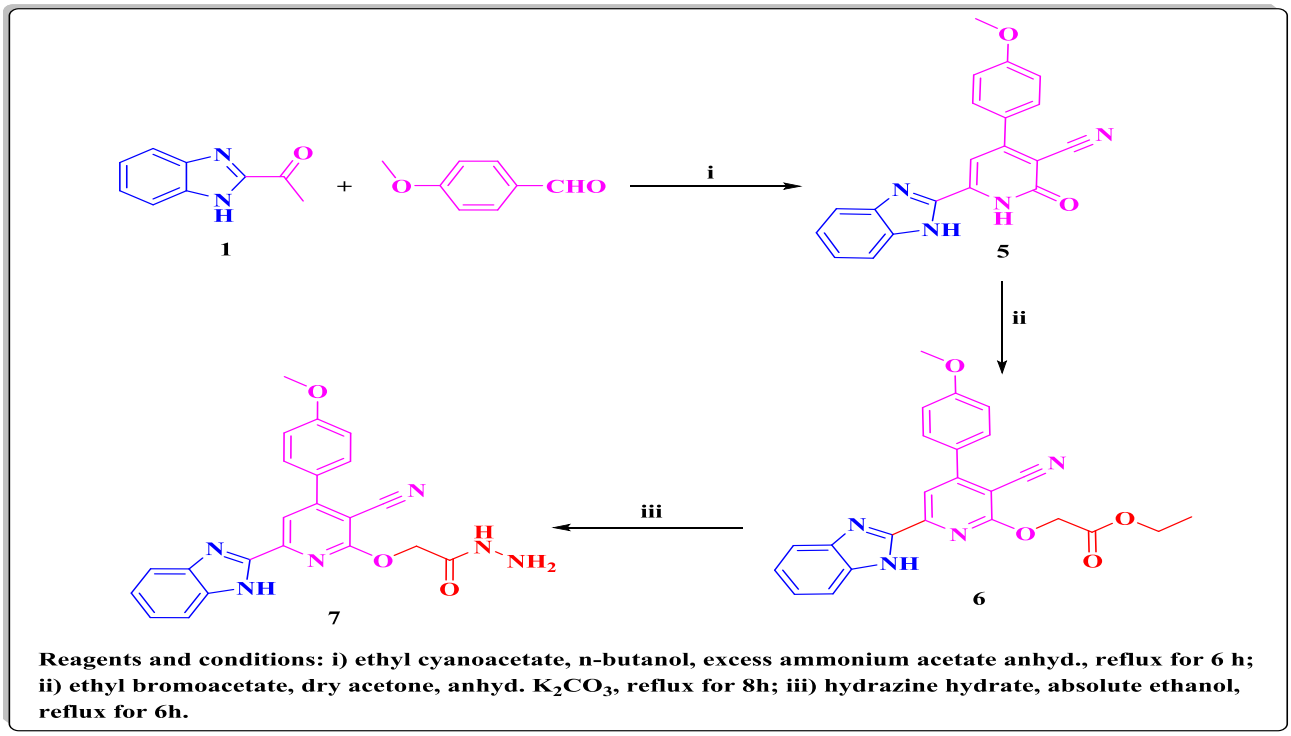
The pathways utilized for synthesis of the target compounds **3–11** were depicted in (**Schemes 1-3)**. The key starting compound 1-(1*H*-benzo[*d*]imidazol-2-yl) ethan-1-one **(1)** was prepared according to reported method (44). Compound **1** was condensed with dimethylformamide-dimethyl acetal (DMF-DMA) to afford N-dimethylenaminone derivative **2** [45]. Compound **2** was refluxed with the respective primary aromatic amine (aniline derivatives or sulfonamide derivatives) in acetic acid to give the corresponding target N-aryl enaminones **3a–d** and **4a–c (Scheme 1)**. The separated compounds obtained as a mixture of *Z* and *E* isomers as indicated from NMR spectra. IR spectra of compounds **3a-d** and **4a-c** revealed well-defined bands at 3362-3122 and 1650-1636 cm^{-1} referring to NH and C=O groups, respectively, whereas, the sulfonyl group of compounds **4a-c** were presented as characteristic bands at the region 1279-1095 cm^{-1}. Moreover, ^{1}H NMR spectra of **3a–d** and **4a-c** revealed the presence of a pair of doublets at δ 6.40- 7.01 ppm corresponding to the *Z* and *E* olefinic proton (–COC<u>H</u>=). The other olefinic proton (=C<u>H</u>-NH-) was overlapped with the aromatic protons at δ 7.05–8.52 ppm. The enaminones NH proton appeared as exchangeable downfield pair of doublets at δ 10.29 - 11.98 ppm. On the other hand the NH protons of benzimidazole nucleus appeared downfield singlet signal at δ 12.99 - 13.19 ppm due to intramolecular hydrogen bonding with carbonyl group of enaminone moiety. Moreover the ^{1}H NMR spectra of compounds **4a-c** revealed additional exchangeable signals at δ 11.42–11.98 ppm due to SO_2NH proton. The protons of methoxy **3b**, acetyl **4a**, methyl **4b** and dimethyl groups **4c** appeared as a singlet signals at δ 3.77, 1.92, 1.94 and 2.23 ppm, respectively. ^{13}C NMR spectra of compounds **3a-d** and **4a-c** showed different signals at δ 93.13-145.30 ppm corresponding to the olefinic and aromatic carbons. A signal at δ 180.00 - 184.00 ppm is attributed to C=O group, in addition, the spectra revealed signals at δ 55.80, 22.98, 23.0, 23.37, 26.31 related to the methoxy, acetyl and dimethyl carbons of compounds **3b**, **4a**, **4b**, **4c** respectively.

One-pot multi–component reaction of 2-acetylbenzimidazole **(1),** ethyl cyanoacetate, *p*-methoxybenzaldehyde and an excess amount of anhydrous ammonium acetate as a source of ammonia led to the formation of the corresponding pyridone derivative **5** in a good yield (46). Moreover, refluxing the later compound with ethyl bromoacetate in dry acetone and in the presence of anhydrous potassium carbonate afforded the corresponding ester derivative **6**. Hydrazinolysis of **6** with hydrazine hydrate in refluxing ethanol gave the corresponding pyridin-2-yloxyacetohydrazide derivative **7 (Scheme 2)**. The structures of compounds **6** and **7** were confirmed by microanalytical and spectral data. IR spectrum of compound **6** revealed well defined bands at 3303, 2220, 1666 cm^{-1} attributed to the functionalities NH, CN, C=O groups respectively. Furthermore, ^{1}H NMR spectrum of **6** represented the characteristic triplet-quartet pattern of the ethyl group at δ 1.20, 4.20 ppm, a singlet at δ 3.83 ppm due to -OCH_3 group. And benzimidazole-NH proton appeared as singlet at 12.70 ppm. The aromatic protons appeared at their expected regions. Its ^{13}C NMR spectrum showed upfield signals at δ 14.40, 55.94, 61.64, ppm corresponding to CH_3, OCH_3, CH_2 groups respectively. The signals of the aromatic carbons appeared at the expected region, in addition to the signal at δ 168.77 ppm due to C=O ester group. IR spectrum of pyridin-2-oxyacetohydrazide **7** showed the appearance of peaks at 3254, 3413cm^{-1} confirming the presence of the hydrazide -NH-NH_2 moiety. The ^{1}H NMR spectrum confirmed the presence of the hydrazide -NH-NH_2 group as two exchangeable singlet signals at δ 4.55, 9.37 ppm

corresponding to NH_2 and NH protons respectively. Additionally, ^1H, ^{13}C NMR spectra of **7** lacked the signals related to the ethyl ester moiety.



Scheme 1: Synthesis of benzimidzol-enaminone derivatives **3a-d** and **4a-c**



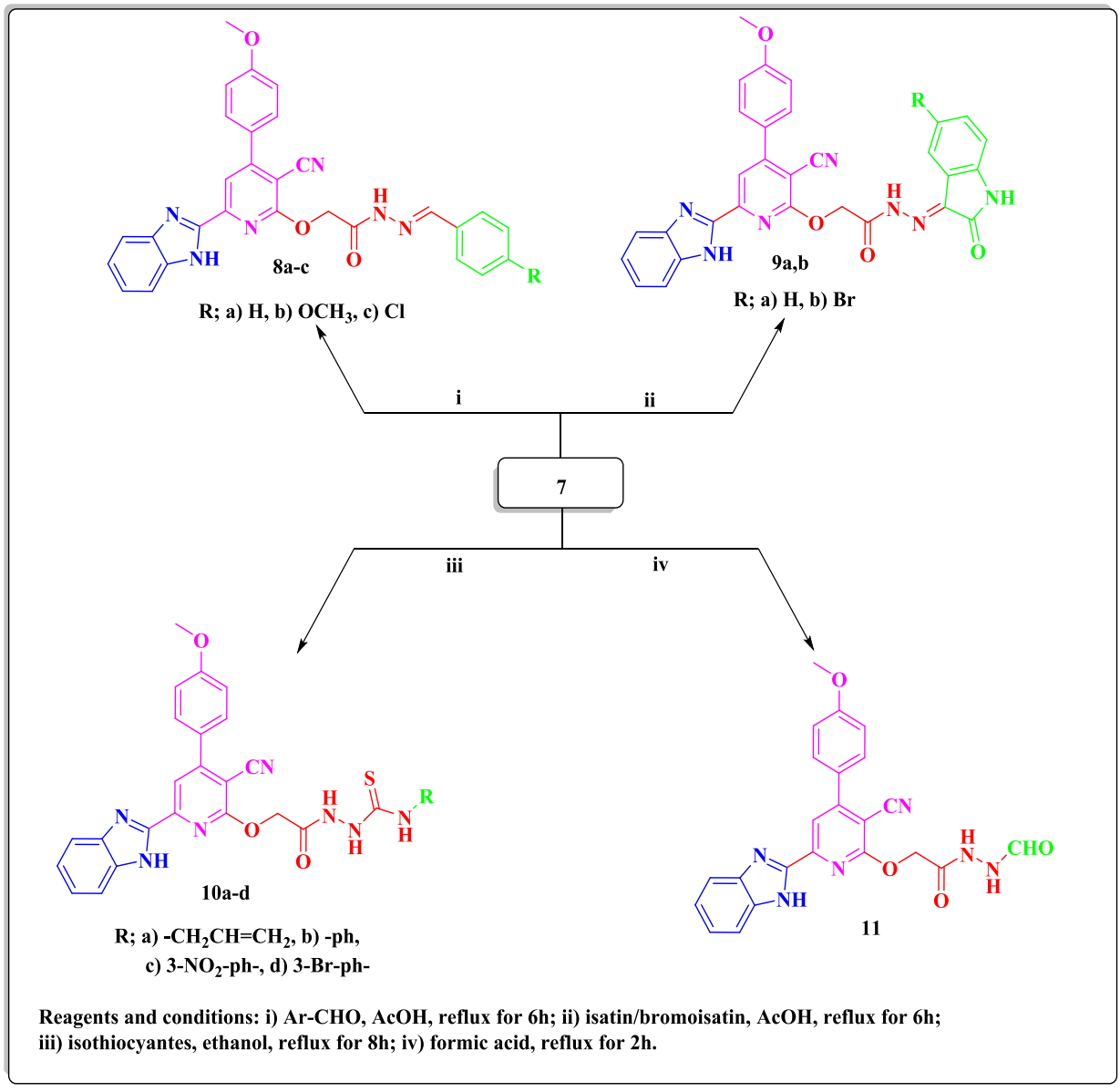
Scheme 2: Synthesis of pyridin-2-oxyacetohydrazide **7**

Furthermore, the acetohydrazide derivative **7** was utilized as a convenient precursor for the preparation of different acetohyrazones. Reaction of compound **7** with various aromatic aldehydes or isatins in boiling ethanol and in the presence of a catalytic amount of glacial acetic acid afforded the hydrazones **8a-c** and **9a,b** respectively in good yields **(Scheme 3)**. ^1H NMR spectra of hyrazones **8a-c** represented a singlet signal at δ 8.27 - 8.33 ppm due to the azomethine proton (CH=N). An extra singlet signal at δ 3.89 ppm related to *p*-OCH_3 protons was observed in H NMR spectrum of compound **8b**. IR spectra of compounds **9a,b** revealed absorption bands at the range 1668-1640 cm^{-1} attributed to C=O groups of hydrazide moiety and the newly conjugated indolin-2-one ring.

Another evidence for the presence of indoline ring is the appearance of a signal at δ 175.48 ppm in ^{13}C NMR spectrum corresponding to C=O group of indolin-2-one.

The thiosemicarbazide derivatives **10a-d** was also obtained by the treatment of the parent acetohydrazide **7** with different isothiocyanates in refluxing DMF **(Scheme 3)**. The structures of the products **10a-d** were confirmed from microanalytical and spectral data. The disappearance of the exchangeable signal at δ 4.55 ppm (NH_2) and appearance of four exchangeable signals in the downfield region (3NH of thiosemicarbazide moiety and NH of benzimidazole nucleus) confirmed the formation of products **10a-d**. ^{1}HNMR spectrum of compound **10a** exhibited a doublet at δ 4.19 ppm contributing to (-HN-C$\underline{H}_2$), and two multiplets at δ 5.07, 5.10 ppm due to (=C$\underline{H}_2$), and one multiplet at 5.83 ppm for (C$\underline{H}$=CH$_2$)). Also the ^{1}HNMR spectrum of compounds **10b-d** revealed the presence of phenyl protons in the aromatic region. Another evidence for the formation of thiosemicarbazide compounds **10a-d** is the appearance of signal at δ 180.22-188.22 ppm in ^{13}C NMR spectrum due to C=S group.

Furthermore, the acetohydrazide derivative **7** was refluxed with formic acid to afford the corresponding N-formylacetohydrazide derivative **11**. IR spectrum of compound **11** exhibited two absorption bands at 1686, 1670 cm^{-1} related to 2C=O moieties. ^{1}H NMR displayed the presence of aldehydic proton as a singlet signal at δ 8.22 ppm and disappearance of signal corresponding to NH_2 group. ^{13}C NMR spectrum revealed two signals at δ 169.00, 181.98 contributing to the parent C=O group and the aldehydic carbon (-$\underline{C}$HO) respectively.



Scheme 3: Synthesis of compounds **8a-c, 9a,b**, **10a-d** and **11**

In vitro antimicrobial screening

All the synthesized benzimidazole derivatives **3-11** were screened *In vitro* for their antibacterial and antifungal activities using agar diffusion method [47,48]. The investigated bacterial strains involved three Gram positive, *S. aureus (ATCC 25923), B. subtilis (ATCC 9372)* and *B.cerrus (MT CC 1305),* two Gram negative, *E. coli (ATCC 25922)* and *P. aeruginosa (ATCC® 27953*) in addition to fungal culture *C. albicans (NRRL Y-477).* The antimicrobial activity has been primarily evaluated as the observed growth inhibition zones (mm) resulting from 100 µl of stock solution (10 mg/ml) of the tested compounds and reference drug standards. **(Table 1)** Minimum inhibitory concentrations (MIC µg/mL) were then determined for compounds exhibiting significant growth inhibition zones ≥ 16mm using two-fold serial dilution method [49,50] **(Table 2)**. Also, **(Fig**s. **3A**, **3B**) illustrate the results of zone of inhibition of the tested compounds comparing to reference standards and the MIC are given in **(Figs. 4A, 4B).**

The overall results revealed a significance of antibacterial activity of most of the tested compounds against Gram-positive, Gram-negative bacterial strains comparing with the reference drugs, vibramycin and ciprofloxacin. The enaminones derived from sulfacetamide **4a** and sulfadiazine **4c** exerted excellent and broad spectrum activity in this series with zone of inhibition ~ 18-20 mm comparable to vibramycin and ciprofloxacin (~ 20-25mm). Meanwhile the hydrazide-hydrazone analog **8a** and thiosemicarbazide derivative **10c** revealed good activity (~ 16-17 mm and 16-18 mm respectively) against all tested Gram positive and Gram negative bacterial strains. Compounds **3a, 3b** and **3d** showed limited antimicrobial activity against certain strains (~13-15mm), while compound **3c** showed no activity against all the tested bacterial strains under the screening condition. The enaminone derived from sulfisoxazole **4b** displayed limited and selective activity against Gram positive strains only (~13-14 mm). However, transformation of the ester **6** to its acetohydrazide **7** revealed no significant deference in zone of inhibitions against Gram positive and Gram negative strains. Regarding the effect of the substituents at phenyl ring of the hydrazone derivatives **8a-c**, the results revealed that a significant growth inhibition has been attained by the unsubstituted benzylidene **8a**. Meanwhile the presence of either electron donating or electron withdrawing group OCH_3 or Cl decreased the activity significantly compounds **8b** and **8c** respectively. On the other hand, investigation of hydrazone series derived from isatins **9a,b** it was observed that, the 5-bromoisatin analog **9b** displayed promising anitbacterial activity against Gram negative strains comparing to non-brominated derivative **9a** which showed no activity against the same tested strains. Regarding the influence of the substituent at N-terminal of thiosemcarbazide derivatives **10a-d,** the results revealed that N-allyl analog **10a** showed limited antibacterial activity against Gram positive strains and no activity against Gram negative bacteria was observed. Replacement of the allyl moiety by un/substituted phenyl moiety enhanced the activity. Meanwhile, the substituted phenyl analogs NO_2, **10c** and Br, **10d** exhibiting a significant growth inhibition zones than unsubstituted one **10b**. Comparing the overall order of activity of the later series, it was observed that nitrophenyl > bromophenyl > unsubstituted phenyl > allyl. Moreover N-formyl analog **11** showed moderate activity against Gram positive (~ 14-15 mm) and good activity against Gram negative strains (~ 17 mm).

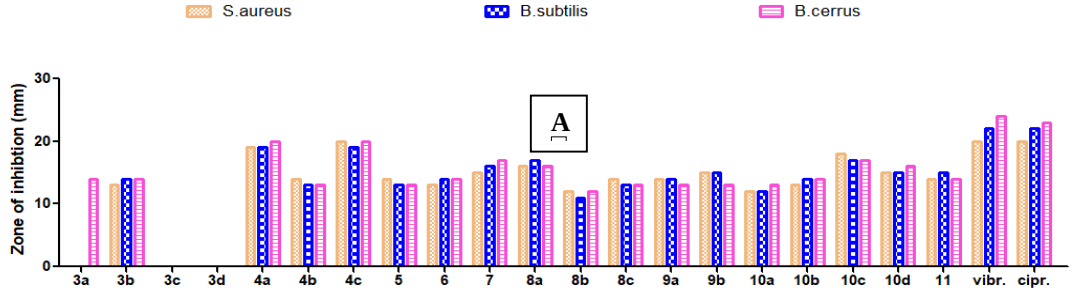
The antifungal results **(Fig. 3c)** revealed that all of the target benzimidazole derivatives displayed antifungal activities against C albicans (NRRL Y-477) except compound **3c**. Some compounds showed good activity **3b, 4c, 6, 7, 8a** and **10b** (zone of inhibition ~16-17 mm) in comparison to ketoconazole (zone of inhibition ~23 mm), and the remaining other compounds revealed moderate activity (zone of inhibition ~ 13-15 mm).

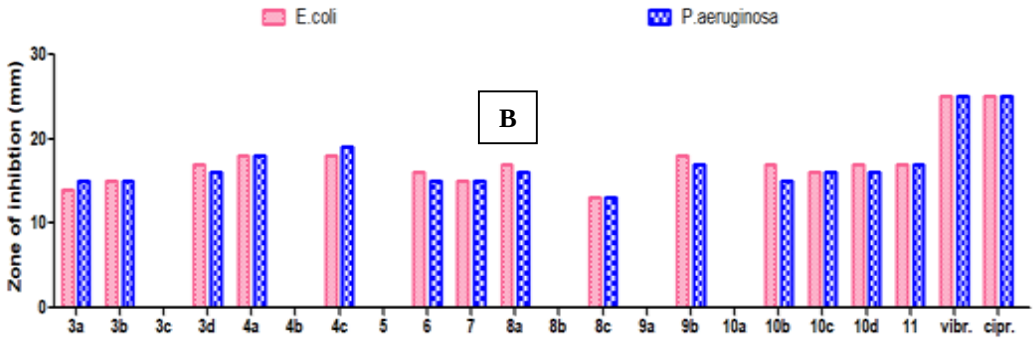
MIC values were determined for the tested compounds that showed zone of inhibition ≥ 16 mm **(Figs. 4A, 4B**). The Gram positive bacterial strains, *S. aureus (ATCC-29213)* and *B.cerrus (MTCC 1305)* were the sensitive strains to the tested compounds **4a** and **4c** more than compounds **8a** and **10c.** Meanwhile Gram positive *B. subtilis (ATCC 9372)* and Gram negative bacterial strains *E. coli (ATCC 25922)* and *P. aeruginosa (ATCC® 27953*) were less sensitive to all the tested compounds. Compound **4c** showed no inhibition under the screening condition. On the other hand, the fungal culture *C. albicans (NRRL Y-477)* was sensitive only to compound 8a (MIC =100 µg/mL). The enaminone derivatives **4a** and **4c** were the most active antibacterial compounds against B.Cerrus Both compounds were more potent against *B.cerrus (MTCC 1305*) than vibramycin (MIC = 50 µg /mL, MIC vibramycin = 62 µg /mL) and 3.3 folds less potent than ciprofloxacin (MIC = 15 µg /mL). Additionally, compound 4c has MIC = 50 µg /mL against *S. aureus (ATCC-29213),* more potent than vibramycin (MIC = 62 µg /mL) and revealed about half the activity of ciprofloxacin (MIC = 31 µg /mL). The hydrazide-hydrazone derivative **8a** and the thiosemicarbazide analog **10c** were the least active antibacterial compounds against all the tested bacterial strains (MIC = 100-150 µg /mL) comparing with the reference drugs.

Table 1 : Antimicrobial activity expressed as the average diameter of inhibition zones (mm) of the chemical compounds against different Gram +ve, Gram −ve bacteria and fungi.

Mean diameter of inhibition zones (Mean ± SEM) (mm)						
	Gram +ve Bacteria			Gram -ve Bacteria		Fungi
Compound No.	***Staphylococcus aureus ATCC 29213***	***B. subtilis ATCC6633***	***B. Cerrus MTCC 1305***	***E. coli ATCC 2592***	***Pseudo-monas aeruginosa ATCC® 27953***	***Candida Albicans* NRRL Y-477**
3a	N. A	N. A	14±0.14	14±0.46	15±0.40	13±0.21
3b	13±0.11	14±0.24	14±0.43	15±0.15	15±0.11	16±0.22
3c	NA	NA	NA	NA	NA	NA
3d	N.A	N.A	N.A	17±0.26	16±0.30	14±0.14
4a	19±0.12	19±0.13	20±0.12	18±0.16	18±0.21	15±0.61
4b	14±0.24	13±0.21	13±0.14	N. A	N. A	13±0.23
4c	20±0.26	19±0.41	20±0.11	18±0.16	19±0.19	17±0.06
5	14±0.15	13±0.33	13±0.18	N. A	N. A	13±0.20
6	13±0.11	14±0.11	14±0.12	16±0.24	15±0.22	16±0.28
7	15±0.15	16±0.12	17±0.11	15±0.32	15±0.12	17±0.15
8a	16±0.13	17±0.12	16±0.22	17±0.22	16±0.32	16±0.20
8b	12±0.15	11±0.13	12±0.15	N.A	N.A	13±0.31
8c	14±0.32	13±0.22	13±0.15	13±0.03	13±0.33	14±0.01
9a	14±0.11	14±0.21	13±0.20	N.A	N.A	14±0.21
9b	15±0.18	15±0.20	13±0.26	18±0.22	17±0.20	13±0.23
10a	12±0.20	12±0.36	13±0.01	N.A	N.A	13±0.14
10b	13±0.02	14±0.10	14±0.21	17±0.11	15±0.12	16±0.33
10c	18±0.33	17±0.15	17±0.23	16±0.12	16±0.10	13±0.31
10d	15±0.20	15±0.22	16±0.24	17±0.20	16±0.32	13±0.32
11	14±0.15	15±0.24	14±0.21	17±0.15	17±0.17	14±0.13
Vibramycin	20	22	24	25	25	N.A.
Ciprofloxacin	20	22	23	25	25	N.A.
Ketoconazole	N.A	N.A	N.A	N.A	N.A	23.7 ± 0.10

NA: No activity under the screening conditions; SEM = standard error mean; each value is the mean of different values.





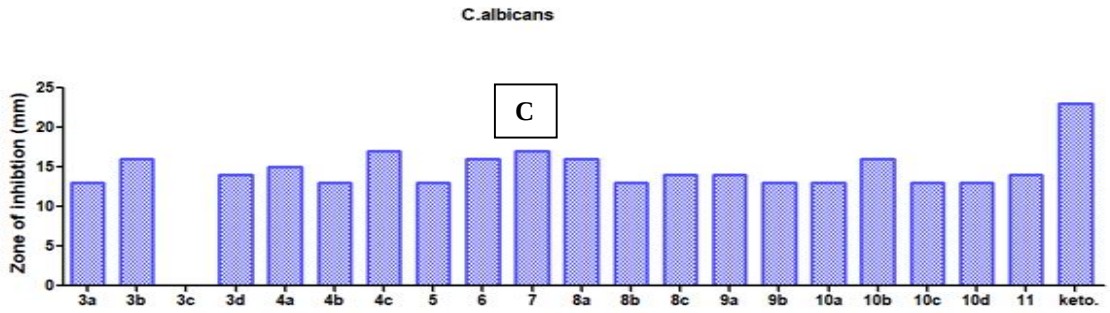
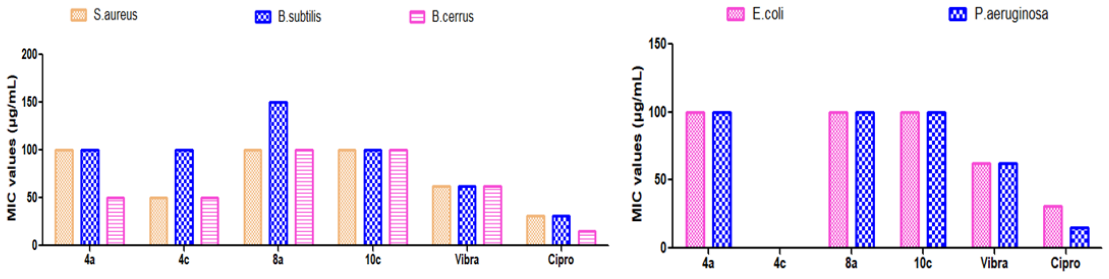


Fig. 3: Antimicrobial activity (zone of inhibition in mm) of the target compounds against (**A**) three strains of Gram positive bacteria and (**B**) two strains of Gram negative bacteria (**C**) Candida albicans fungal strain.

Table 2 :MIC values of the most active target compounds against various microbial strains

	Gram +ve Bacteria			Gram -ve Bacteria		Fungi
Compound No.	***Staphylococcus aureus ATCC 29213***	***B. subtilis ATCC6633***	***B. Cerrus MTCC 1305***	***E. coli ATCC 2592***	***Pseudomonas aeruginosa ATCC® 27953***	***Candida Albicans* NRRL Y-477**
4a	0.10	0.10	0.05	0.10	0.10	0.15
4c	0.05	0.10	0.05	N.A	N.A	N.A
8a	0.10	0.15	0.10	0.10	0.10	0.10
10c	0.10	0.10	0.10	0.10	0.10	N.A
Vibramycin	0.062	0.062	0.062	0.062	0.062	N.A
Ciprofloxacin	0.031	0.031	0.015	0.031	0.015	N.A
Ketoconazole	N.A	N.A	N.A	N.A	N.A	0.062

NA: No activity under the screening conditions



A **B**

Fig. 4: MIC values (µg/mL) of the most active target compounds against (**A**) three strains of Gram positive bacteria and (**B**) two strains of Gram negative bacteria

DNA gyrase and topoisomerase IV inhibitory activity

DNA gyrase supercoiling and topoisomerase IV decatenation assays

The observed promising antibacterial profile of compounds **4a**, **4c**, **8a** and **10c** inspires evaluation of their potential activity (IC_{50}) against target enzymes, DNA Gyrase and topoisomerase ***IV***. They were screened for their efficacy to inhibit DNA gyrase supercoiling (S. aureus DNA gyrase) and topo IV decatenation (*S. aureus Topo IV*) according to reported methods [51,52]. Ciprofloxacin and novobiocin were utilized as reference standards. The results **(Table 3)** indicated that hydrazide-hydrazone analog **8a** revealed inhibition of DNA gyrase supercoiling at a micromolar level that is approximately equipotent to ciprofloxacin (IC_{50} = 0.443 µM, IC_{50} Ciprofloxacin = 0.425 µM), but 1.6

folds less than that obtained by novobiocin (IC_{50} = 0.271μM). Furthermore, the suppression effect of the thiosemicarbazide derivative **10c** (IC_{50}= 0.941 μM) appeared to be 2 and 3.4 folds less than ciprofloxacin and novobiocin respectively. Both sulfacetamide and sulfadiazine analogues displayed weak supercoiling inhibition activity (IC_{50} values 1.774, 3.227 μM, respectively). Interestingly, it has been observed that **8a** also exhibited topo IV decatenation suppression activity (IC_{50}= 1.15 μM) more than ciprofloxacin (IC_{50}= 1.31 μM), whereas less than that obtained by the reference novobiocin by 1.9 times (IC_{50} = 0.6 μM). Compounds **4a**, **4c** and **10c** exhibited weak decatenation suppression activity IC_{50} values; 3.01, 8.04, 2.13 μM, respectively.

DNA gyrase and topoisomerase IV ATPase assays

The target compounds **4a**, **4c**, **8a** and **10c** were further tested by DNA gyrase and topoisomerase IV ATPase assays [53]. And the results were depicted in **(Table 3)** revealed that the enaminone **4a** was the most potent dual inhibitor with IC_{50} value of 0.39 μM against DNA gyrase ATPase and 0.52 μM against DNA topoisomerase IV ATPase. Replacement of the acetamide moiety of compound **4a** with heteroaromatic diazine ring compound **4c** a detectable decrease in inhibitory activity against both DNA gyrase and topoisomerase IV ATPase (IC_{50} = 3.22, 8.04 μM, respectively) was observed.

Compound **8a** was 1.7 folds (IC_{50}= 0.28 μM) more potent than ciprofloxacin (IC_{50}= 0.48μM) against topoisomerase IV ATPase. On the other hand compound 8a revealed moderate ATPase inhibition (IC_{50}= 0.66 μM) against DNA gyrase in comparion with ciprofloxacin and novobiocin (IC_{50} = 0.40, 0.38 μM, respectively). Regarding compound **10c,** it exhibited equipotent inhibition of DNA topoisomerase IV ATPase (IC_{50}= 0.46 μM) as ciprofloxacin (IC_{50}= 0.48μM). Meanwhile it showed 3 folds lower inhibitory activity (IC_{50}= 1.11μM) than both reference drugs ciprofloxacin (IC_{50}= 0.40μM) and novobiocin (IC_{50} = 0.38 μM).

In vitro cytotoxicity assay

The safety profile of the derivatives **4a** and **8a** was evaluated by assessment their cytotoxic effect against normal human cell WI38 using colourmetric cell proliferation MTT assay [54-56]. Both compounds have demonstrated safety profiles much better than that of the reference drugs ciprofloxacin and novobiocin (IC_{50}=138, 179 μM, respectively, IC_{50} Ciprofloxacin; 63.3I μM, IC_{50} Novobiocin; 86.2 μM **(Table 3)**.

Molecular modeling study

Study was performed to understand the mechanistic aspects of the observed antibacterial activity of the selected compounds, **4a, 4c, 8a, 10c** and to explore their binding with the target protein. These compounds were docked into the ATP binding sites co-crystallized *S.aureus* DNA gyrase B (PDB code 4URO) and *S.pneumonia* topoisomerase IV parE (PDB code 1S14) retrieved from protein data bank (RCSB PDB) using MOE 2014.0901software. The molecular docking results of the best fitted conformer are illustrated in **(Tables 4,5)** and presented in **Figs (5–14).** Most of the investigated compounds were well-fitted into the ATP-binding pockets of DNA gyrase B as well as topoisomerase IV parE and showing binding patterns almost similar to that described for the majority of gyrase B and topoisomerase IV inhibitors.

Molecular docking into ATP-binding pocket of DNA gyrase B

In the present investigation, the proposed molecular docking algorithm was initially validated by self-docking of the cocrystallizled ligand (Novobiocin) to the active site DNA-gyrase. The validation results of novobiocin revealed a score energy of –15.86 kcal/mol and RMSD 0.34 A°. The docking pose achieved interactions with the key amino acids in the active site through hydrogen bonds Asp81, Asp89, Asn54 and Arg144 **(Fig.5)**. The synthesized benzimidazole derivatives **4a, 4c, 8a** and **10c** were docked at the same ligand binding site, and the result depicted in **(Table 4)**. The molecular docking results of compound **4a** showed that the oxygen atom of sulfonamide moiety is oriented toward Arg144 amino acid. Moreover, NH group of benzimidazole is involved in hydrogen bond with key amino acid Asn54. This might be contributed to its higher ATPase potency against DNA gyrase **(Fig. 6)**. In contrast, compound **4c** (**Fig. 7**) lack the ability to form hydrogen bond interaction with **Arg144** that might be attributed to the presence of bulky pyrimidine nucleus and consequently decreased its potency compared to **4a**.

Compound **8a** is the most potent gyrase inhibitor among the newly synthesized compounds with approximately identical inhibition as the standard reference, ciprofloxacin. Compound **8a** revealed the first most negative energy score -16.83 kcal/mol with higher predicted binding affinity than the cocrystallized ligand novobiocin`-15.86 kcal/mol, **(Table 4)** compound **8a** was embedded nicely in the active pocket similar to Novobiocin through forming of extensive hydrogen bond network involving carbonyl oxygen with Arg144. Aromatic ring making a bifurcated hydrogen bond with Asp81, double arene – cation interaction was also observed firstly between aryl moiety and key residue Asn54 that stabilized the compound in the receptor while the second between cyanopyridine ring and IIe86 **(Fig. 8)**.

Considering the binding pose of compound **10c** showed a strong hydrogen bond with Arg144**.** Furthermore, the aromatic ring attached to cyanopyridine moiety shared the fixation within the binding pocket through H-bond donor with the side

chain of Asp81. Additionally, arene–cation interaction with Asn54 was noticed with the center of phenyl ring and another hydrogen bond appeared between Gly85 residue and cyanide moiety **(Fig. 9)**.

Table 3: Assessment of DNA gyrase, topoisomerase IV inhibition activity and cytotoxicity for the most promising compounds

	IC_{50} (μM)				
Compound No.	**DNA gyrase[a]**		**TOPO-IV**		**Cytotoxicity WI38[b]**
	Supercoiling assay	**ATPase[a] activity**	**Decatenation assay[a]**	**ATPase[a] activity**	
4a	1.774	0.39	3.01	0.52	138
4c	3.227	1.98	8.04	2.74	43.1
8a	0.443	0.66	1.15	0.28	57.5
10c	0.941	1.11	2.13	0.46	179
Ciprofloxacin	0.425	0.40	1.31	0.48	63.3
Novobiocin	0.271	0.38	0.6	0.19	86.2

[a]S. aureus, [b] normal human cell line (fibroblasts derived from lung tissue).

Table 4: Molecular docking results of compounds **4a**, **4c**, **8a** and **10c** into DNA gyrase active site

Compound No.	**Docking score (kcal/mol)**	**Interacting residues (Bond Length Aº)**	**Atoms of compounds**	**Binding interaction**
4a	-11.87	Asn54 (3.23)	NH(Benzimidazole ring)	H-donor
		Arg144 (3.48)	$O(SO_2)$	H-donor
		Arg144 (3.44)	$O(SO_2)$	H- donor
		lle86(4.31)	Phenyl(Sulfa)	Arene–H
4c	-5.82	Asn54 (3.51)	Pyrimidine ring	Arene–H
		Gly85 (3.34)	$O(SO_2)$	H-donor
		Pro87 (4.40)	Phenyl(Sulfa)	Arene–H
8a	-16.83	Asp81 (3.24)	Phenyl	H-donor
		Asp81 (3.54)	CH_3(Methoxy)	H-donor
		Asn54 (3.41)	Phenyl	Arene–H
		Arg144(2.97)	O(CONH)	H-donor
		lle86(4.69)	Cyanopyridine ring	Arene–H
10c	-10.02	Asp81 (3.03)	Phenyl	H-donor
		Asn54 (3.58)	Phenyl	Arene–H
		Arg144 (2.23)	O(CONH)	H-acceptor
		Gly85 (2.83)	N(CN)	H-acceptor
		Asn145 (3.07)	$O(NO_2)$	H-acceptor
Novobiocin	-15.86	Asp81 (2.79)	H ($OCONH_2$)	H-donor
		Asp89 (2.63)	O(OH)(phenyl)	H-donor
		Asn54 (3.41)	O(OH)(phenyl)	Arene–H
		Arg144 (2.97)	O(CO)(Coumarin scaffold)	H-acceptor
		lle84 (3.90)	O(CO)(Coumarin scaffold))	Arene–H

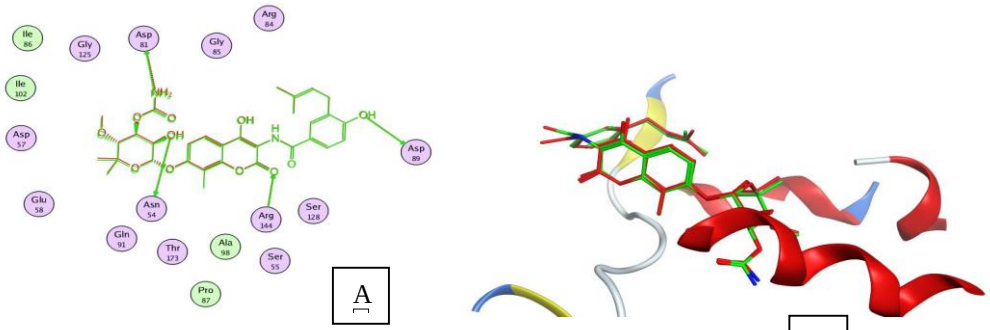


Fig. 5: 2D diagram **(A)** and 3D representation **(B)** of the superimposition of the docking pose (green) and the co-crystallized ligand Novobiocin (red) in the *S.aureus* DNA gyrase B active site (PDB ID: 4URO)

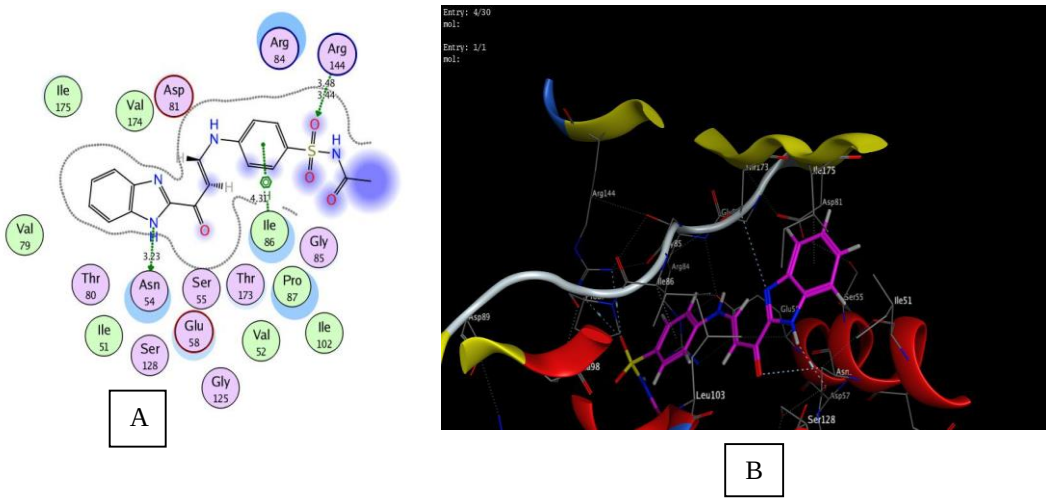


Fig. 6: 2D (a) and 3D (b) diagrams of compound **4a** into the ATP binding site of DNA gyrase B enzyme

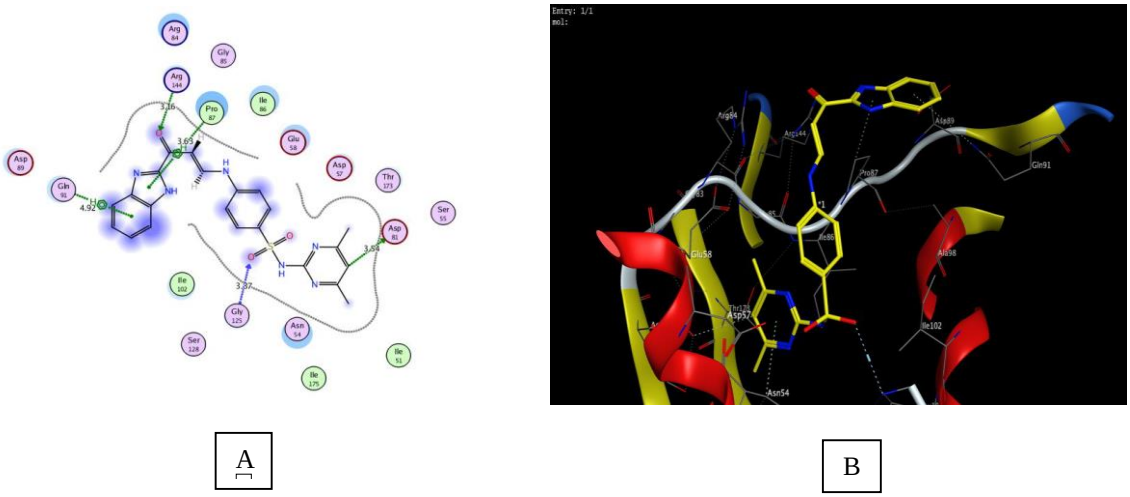


Fig. 7: 2D (a) and 3D (b) diagrams of compound **4c** into the ATP binding site of DNA gyrase B enzyme

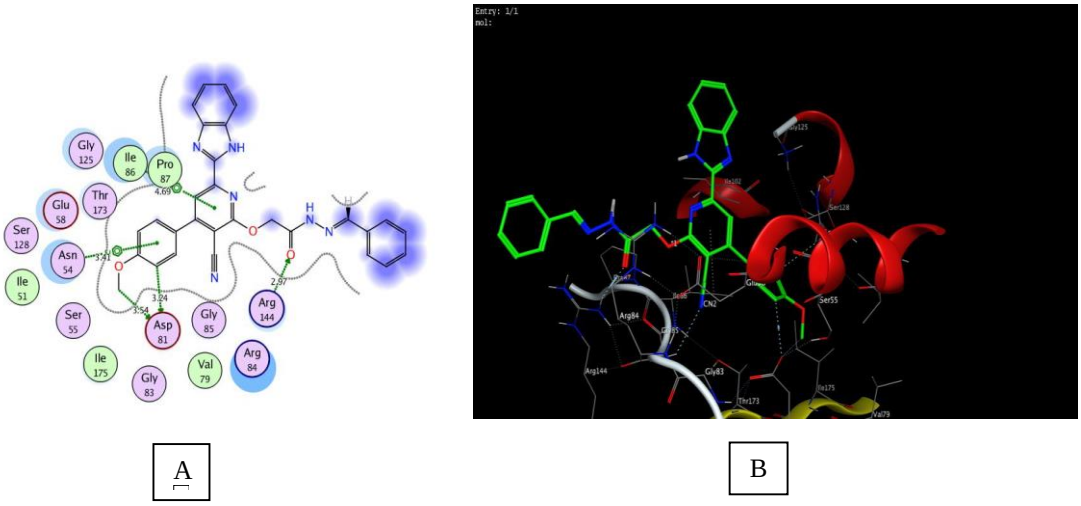


Fig. 8: 2D (a) and 3D (b) diagrams of compound **8a** into the ATP binding site of DNA gyrase B enzyme

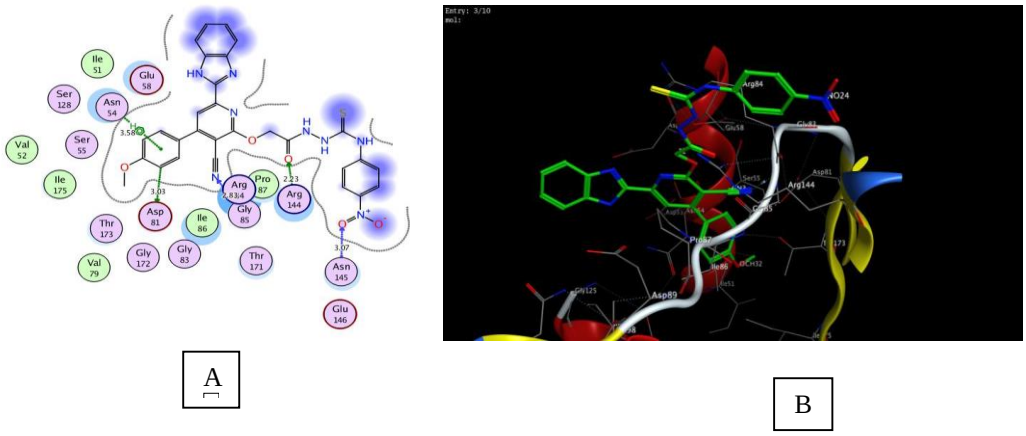


Fig. 9: 2D (a) and 3D (b) diagrams of compound **10c** into the ATP binding site of DNA gyrase B enzyme

Docking into ATP-binding pocket of topoisomerase IV parE

At the beginning validation of the molecular docking results was confirmed by redocking of the cocrystallized ligand novobiocin in the active site of ATP-binding site of topoisomerase IV parE. The result revealed a score energy of –6.25 kcal/mol **(Table 5)** and a small RMSD value (0.28 A^o) between the experimental co-crystallized ligand pose and the docked pose (Fig **10**). The results also displayed the capability of the docking pose to reproduce all the hot spot interactions achieved by the cocrystallized ligands in the active site with Asn1042**,** Asp1069**,** Asp1077 and Arg1132. The molecular docking results of compounds **4a, 4c, 8a,** and **10c** presented at table 3. Compound **4a** showed 5 folds improvement in potency with IC_{50} = 0.52µM than compound **4c** with IC_{50} = 2.74µM, this might be attributed to the probably of sulfacetamide moiety to bind with two important amino acids Asn1042, Asp1069 through hydrogen bond acceptor. Arene – hydrogen interaction and hydrogen bond interaction were also observed between imidazole ring and carbonyl group with Arg1072 and Arg1132 respectively **(Fig 11)**. On the other hand, compound **4c** revealed very poor interaction through arene –H interaction with Asn1042 and undesirable weak interaction between pyrimidine and imidazole nucleus with other amino acids, Val1039, IIe1090, Thr1163 **(Fig 12)** this might be the reasons for the in inhibitory activity of compound **4c**.

Compound **8a** showed an approximately 2-folds (IC_{50} = 0.28 µM) increased in topoisomerase IV activity relative to the standard ciprofloxacin with IC_{50}=0.48 µM, the docking conformation interprets the remarkable potency of compound **8a** might be contributed to its optimum size to fit into the region of the ATP binding site and/or was able to maintain the required planarity with minimum degree of torsion. Also, it showed lowest energy-based scoring function (-9.29 Kcal/mol) even superior to the cocrystallized ligand novobiocin (-6.25 Kcal/mol) verifies its best fitting into the active site topoisomerase IV. In addition compound **8a** revealed strongly interacts through hydrogen bond donor and acceptor with Arg1132, Asp1069 as well as the amino acid Gly1073 , and the compound are stabilized in the receptor through *arene-H* interaction with Asn1042 **(Fig.13)** that resembles novobiocin interaction in binding with topoisomerase IV active site.

Regarding the docking model and biological data of compound **10c,** the nitro moiety resulted in good activity profile with IC_{50} = 0.46 µM nearly equipotent to standard ciprofloxacin (IC_{50}=0.48 µM). The Docking results verified the importance of the f nitro group which formed non-polar interaction with the hydrophobic pockets Val1119, Val1039, Val1165, Leu1091, lle1090 and an additional polar contact with essential Asn1042. The NH group of the thiosemicarbazide moiety allowed hydrogen bond donor with the side chain of Asp1069. Additionally, the aromatic ring group shared the fixation within the binding pocket through arene-H interaction with Arg1132. Moreover, additional hydrogen bonds between sulfur and nitrogen atoms of cyanopyridine were observed with the amino acids, Gly1073, Arg1072 **(Fig14)**. Finally, the biological evaluations and molecular docking study confirm that our docking study are in agreement with the antimicrobial results, we could deduce that the most potent compound **8a** possess the highest binding affinity with dual targeting DNA gyrase B and topoisomerase IV pare.

Table 5: Docking of compounds **4a**, **4c**, **8a** and **10c** into topoisomerase IV active binding site

Compound No.	Docking score (kcal/mol)	Interacting residues (Bond Length A°)	Atoms of compounds	Binding interaction
4a	- 6.12	Asp1069 (2.82)	Phenyl(Benzimidazole)	H-donor
		Asn1042 (3.04)	NH (Benzimidazole ring)	H-donor
		Arg1132 (3.32)	O (Sulfone moiety)	H-acceptor
		Arg1072 (3.40)	O (Sulfone moiety)	H-acceptor
		Met 1074(3.64)	Aromatic (sulfa	Arene–H
4c	-8.04	Asn1042(4.33)	Benzimidazole nucleus	Arene–H
		Val1039 (3.43)	Benzimidazole nucleus	Arene–H
		Val1039 (3.48)	Pyrimidine ring	H-donor
		Ile1090(3.62)	Benzimidazole nucleus	Arene–H
8a	-9. 29	Asp1069 (3.60)	Phenyl	H-donor
		Asn1042 (3.61)	Phenyl	Arene–H
		Arg1132 (3.03)	CO(oxygen)	H-donor
		Gly 1073 (3.12)	N(cyanopyridine)	H-acceptor
10c	-13.03	Asn1042 (3.05)	O(NO_2)	H-donor
		Arg1132 (4.36)	Phenyl	Arene–H
		Arg1072 (3.52)	N(cyanopyridine)	H-donor
		Asp1069 (3,53)	N (thiourea)	H-donor
		Gly1073(4.78)	S (thiourea)	H-acceptor
Novobiocin	-6.25	Asp1077(2.69)	O(OH)(phenyl)	H-donor
		Asn1042 (2.73)	O(OH)(oxan-4-yl)	H-acceptor
		Asp1069 (2.64)	H ($OCONH_2$)	H-donor
		Arg1132 (3.11)	O(CO)(Coumarin)	H-donor
		Arg1132 (3.15)	O(CO)(Coumarin)	H-donor

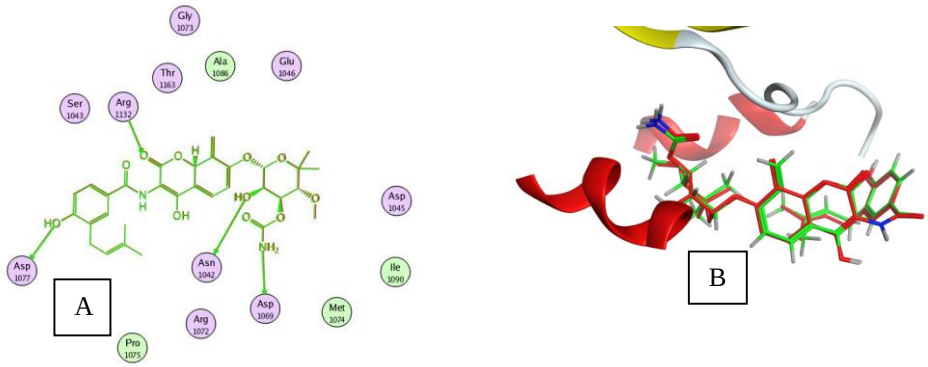


Fig. 10: 2D diagram **(A)** and 3D representation **(B)** of the superimposition of the docking pose (green) and the co-crystallized ligand Novobiocin (red) in the *S.pneumonia*topo IV parE active site (PDB ID: ISI4) with an RMSD of 0.34 A**.**

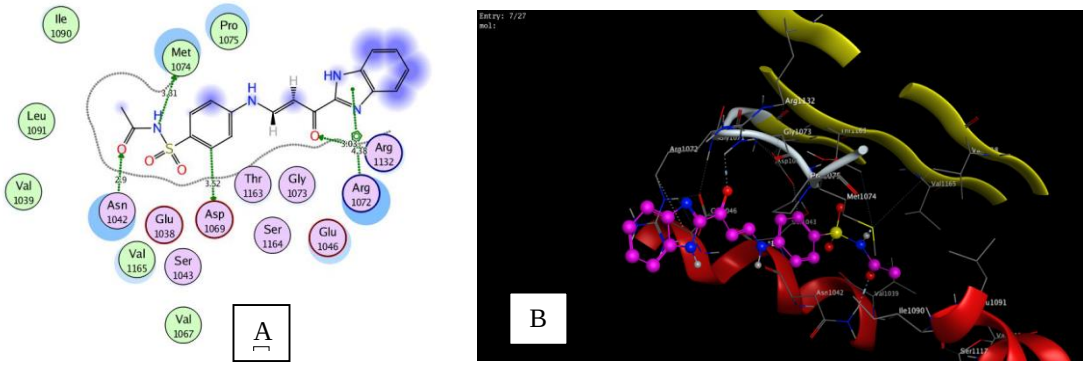


Fig.11: 2D (a) and 3D (b) diagrams of compound **4a** into the ATP binding site of topoisomerase IV pare

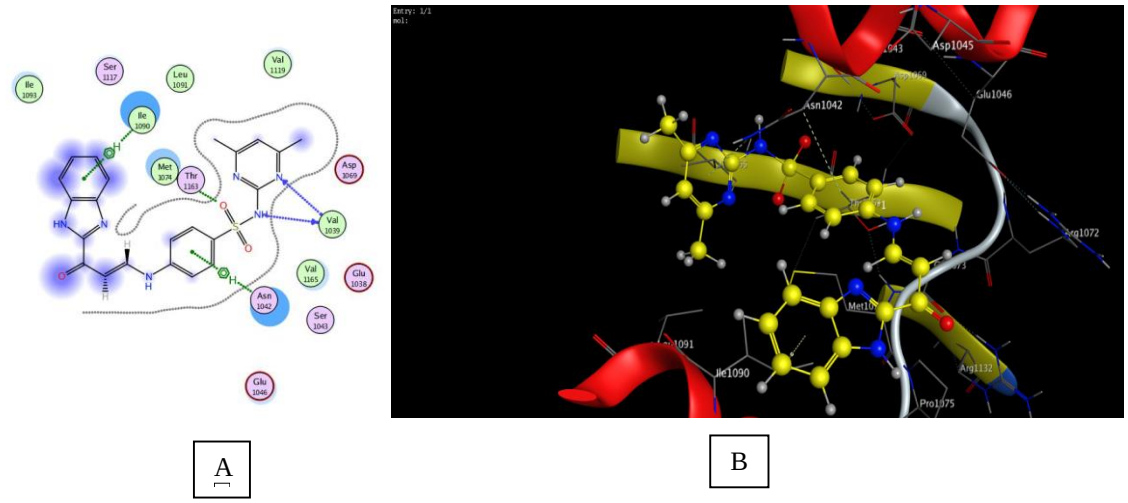


Fig. 12: 2D (a) and 3D (b) diagrams of compound **4c** into the ATP binding site of topoisomerase IV pare

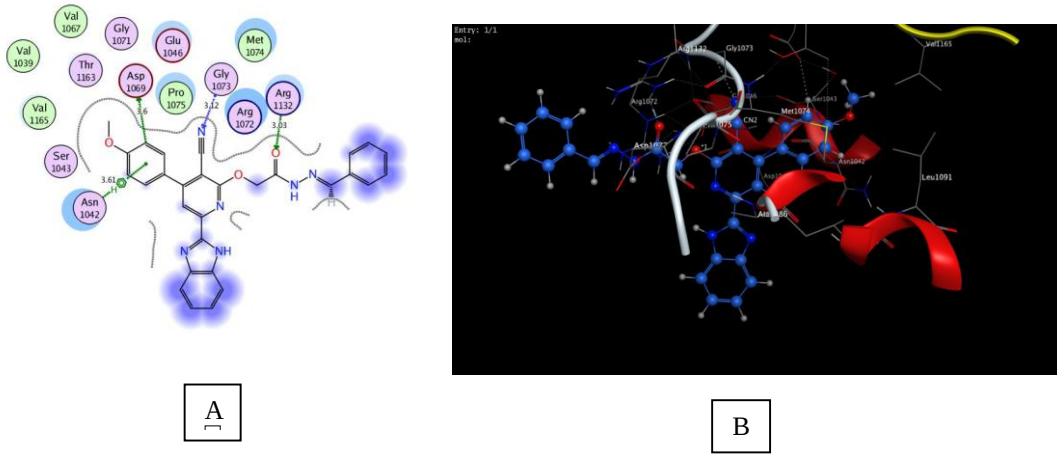


Fig. 13: 2D (a) and 3D (b) diagrams of compound **8a** into the ATP binding site of topoisomerase IV pare

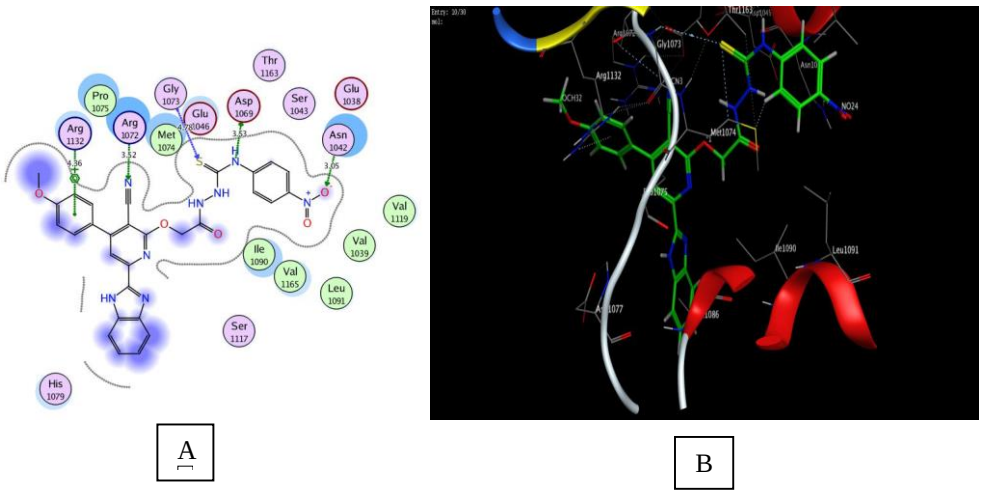


Fig. 14: 2D (a) and 3D (b) diagrams of compound **10c** into the ATP binding site of topoisomerase IV pare

Conclusion

In this study, nineteen new compounds containing benzimidazole scaffold were synthesized, their chemical structure were elucidated and evaluated for *in vitro* antimicrobial activity against Gram positive using agar diffusion method. The results revealed that the compounds showed variable potency against Gram positive, Gram negative bacterial strains and C. albicanis fungus in comparison to the references standard vibramycin, ciprofloxacin and ketoconazole. Minimal inhibitory concentrations were determined for the most promising compounds **4a, 4c**, **8a** and **10c** that exhibited growth inhibition zone ≥ 16mm. DNA-gyrase and topoisomerase IV inhibition assay of the most active compounds (4a, 4c, 8a and 10c) showed that they possess a high to moderate inhibitory effect having IC_{50} range from 0.443 – 3.227 µM/. for DNA gyrase supercoiling assay (ciprofloxacin 0.425 and novobiocin 0.271 µM) and a high to weak inhibitory effect for DNA topo IV decatenation assay, IC_{50} (1.15- 8.04 µM) in comparison to ciprofloxacin 1.31 µM and novobiocin 0.6 µM. For ATPase activity, the compounds showed high to moderate activity in case of DNA gyrase and topo IV, I(C_{50} 0.39 - 1.98 µM and 0.28 – 2.74 µM respectively) comparing with ciprofloxacin 0.40 and 0.48 µM and novobiocin 0.38 and 0.19 µM respectively. Compounds **4a** and **8a** exhibited the most promising activity in DNA gyrase and topoisomerase IN ATPase assays comparing to ciprofloxacin as a standard drug revealing a safer profile against the normal WI38 cells in comparable with both ciprofloxacin and novobiocin. Compound **8a** showed dual inhibitory activity for both DNA gyrase supercoiling and topoisomerase IV decatenation assays (IC_{50} = 0.443 and 0.28 µM respectively). Docking study was performed for the most promising compounds **4a, 4c**, **8a** and **10c** to explore their modes of binding with the amino acid residues. Molecular docking results revealed that the compound 8a preserves the essential binding interaction with DNA–gyrase and topoisomerase IV cleavage complex similar to that observed with the co-crystallized ligand, Novobiocin.

Conflicts of interest

There are no conflicts to declare.

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