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New Benzofuran-Pyrazole Based Compounds as Promising Antimicrobial Agents: Design, Synthesis, Antioxidant, Anti-Inflammatory, DNA Gyrase B Inhibition and In Silico Studies

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Abstract: The alarming rise of antibiotic resistance has made it imperative to find novel antimicrobial medications. This study reports the design and synthesis of new molecules based on benzofuran-pyrazole scaffolds hybridized with various N/O heterocycles using the molecular hybridization process. The newly synthesized candidates were confirmed using micro-analytical and spectral analyses, and their antimicrobial characteristics were assessed against different bacterial and fungal isolates in comparison with novobiocin and clotrimazole as antibacterial and antifungal standards, respectively. In addition, the new compounds were further evaluated as in vitro antioxidants and anti-inflammatory congeners. The most promising broad spectrum antimicrobial compounds 9, 10 with values ranging from 2.50-20.60 µg/mL were further examined as E. coli DNA gyrase B enzyme suppressors using novobiocin as a reference drug. In silico computational studies revealed that compound 9 produced a good fit in the active site of E. coli DNA gyrase B in comparison to novobiocin. Additionally, the drug similarity and ADMET parameters of compound 9 were examined in conjunction with the antibiotic ciprofloxacin, making it a potential candidate for further development as a promising antimicrobial agent.

Keywords: benzofuran-pyrazole scaffold; antimicrobial activity; antioxidant; in vitro anti-inflammatory DNA gyrase enzyme; computational studies

1. Introduction

Bacterial diseases are considered a global threat due to the fact that many bacterial isolates are increasing resistance to many antibiotics. Right now, the globe faces a post-antibiotic period where common bacterial infections and mild injuries could be fatal [1,2].

By 2050, it is predicted that more than 10 million people globally will pass away from multiple antibiotic resistance if this problem is not solved [2]. Different pathogenic microbes such as viruses, yeasts, bacteria, and fungi are frequently the cause of severe infections, which significantly exacerbate suffering in those with weakened immune systems and cause severe morbidity and mortality [3–5]. Antibiotics are effective tools in the fight against pathogenic bacterial by killing or suppression the

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growth of bacteria, but their misuse contributes to the emergence of antibiotic resistance, which has detrimental effects on human health [6].

The emergence and wide-spread distribution of drug-resistant bacteria including multi-drug resistant (MDR) and pan-drug resistant pathogens represent the need of the hour to develop new drugs active against both drug-sensitive and -resistant Gram-positive and Gram-negative pathogens [7–9].

Heterocyclic ring systems are powerful backbones with many biological characteristics due to their similarities to various natural bioactive molecules [10]. Benzofuran is a fundamental structural unit in a variety of biologically active natural and synthesized products [11], covering a wide range of categories such as antiviral, antibacterial, antifungal, antiparasitic, anti-TB, anticancer, anti-Alzheimer's disease, analgesic, anti-inflammatory, and antihyperglycemic activities [12–19].

Recently, numerous derivatives bearing benzofuran nucleus hybridized with different heterocyclic cores have been designed, synthesized and tested for their anti-microbial activities, and some of them showed promising potency. Siddiqui *et al.* represented that the benzofuran-isoxazole hybrids **I** showed potential anti-bacterial activities with inhibition zones of 9–30 mm at a concentration of 1 mg/mL, and some of them were comparable to the reference chloramphenicol (IZ: 21–32 mm) [20]. Whereas, Liu and the coworkers prepared a series of benzofuran-oxazole hybrids, which produced excellent antibacterial activity against a panel of Gram-positive and Methicillin-resistant *S. aureus* (MRSA), as well as Gram-negative bacteria, in comparison to cefotaxime and sodium penicillin, with MICs ranging from 0.78 to 6.25 μg/mL. Specifically, the methanone and imine compounds **II** and **III** showed selective activity against *S. aureus* among the tested strains, with great MIC values: 3.12-12.5 μg/mL [21]. Furthermore, the conjugation of the benzofuran nucleus with indole and/or pyrimidine ring systems as compounds **IV** and **V**, respectively, produced promising broad-spectrum antibacterial activity with MIC values of 3.13–6.25 g/mL and 10.41–18.45 μg/mL, respectively [22,23] **(Figure 1)**.

Moreover, it has been reported that hybridization of benzofuran nucleus with norfloxacin molecular structure generated compound **VI** is a widespread antimicrobial agent. It retained the antibacterial activity of norfloxacin (a widespread antimicrobial agent) and produced better antibacterial activities than streptomycin sulphate and better antifungal activities than norfloxacin [24]. Also, Chougala and his team reported the excellent anti-microbiological effects of the 3-(3-benzofuranyl)-coumarin derivatives **VII** against *S. aureus*, *C. albicans*, and *A. niger*, with MIC values of 0.2–12.5 μg/mL [25] **(Figure 1)**.

Wang and his assistants exhibited that the benzofuran hybrid **VIII** could suppress the pigment production of *S. aureus* Newman and three MRSA strains with IC_{50} values: 0.38–5.45 nM and exhibited good oral bioavailability (F = 42.2%) as well as favorable safety characteristics [26]. In addition, the benzofuran derivatives **IX** exhibited inhibition zones of 11–28 mm at 100 μg/mL against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* in comparison to ciprofloxacin (IZ = 21–28 mm at 100 μg/mL) [27]. Also, it has been documented that the benzofuran derivatives **X** and **XI** showed potential anti-bacterial activities with inhibition zones 19–27 mm at 25 μg/mL, which were comparable to norfloxacin (IZ = 19–25 mm at 25 μg/mL) against the previous tested organisms [28–30] **(Figure 1)**.

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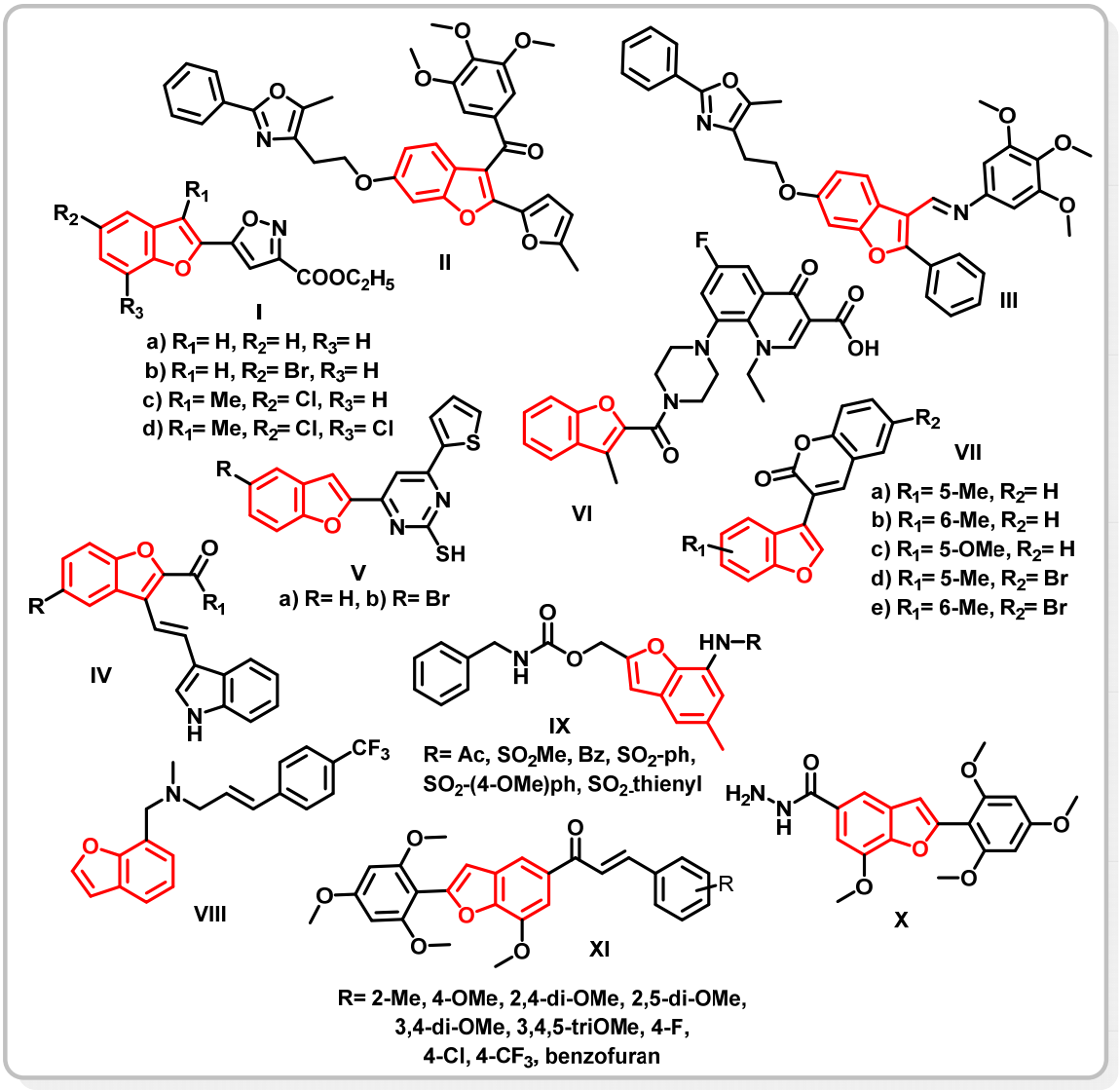

Figure 1. Examples of various *N*/*O* - based heterocycle-benzofuran hybrids.

In medicinal chemistry, the pyrazole ring structure is extensively spotted as a pharmacophore, and the fortification of the modern organic synthesis toolbox demands synthetic tactics to generate its derivatives. Considering the wide range of biological activities of pyrazoles and their presence in naturally occurring compounds, pyrazole has been the topic of research for thousands of researchers all over the world [31–34]. Many studies have focused on the antibacterial action of various pyrazole-based derivatives against drug-resistant bacteria and fungi. The pyrazole nucleus plays an obvious and important role in combating bacterial infections owing to its unique structural features, which are more conducive to the interaction of DNA, enzymes, and receptors [35].

In recent studies, the hybridization of benzofuran ring with pyrazole nucleus has been identified as medically promising agents and constitutes an important scaffold in the field of designing and synthesis of promising antimicrobial candidates. Numerous benzofuran-pyrazole-based derivatives possess an excellent efficacy as antimicrobial agents. **Figure 2** represents various derivatives which are based on benzofuran-pyrazole and/or benzofuran-pyrazoline scaffolds possessing significant antimicrobial activities [36–40].

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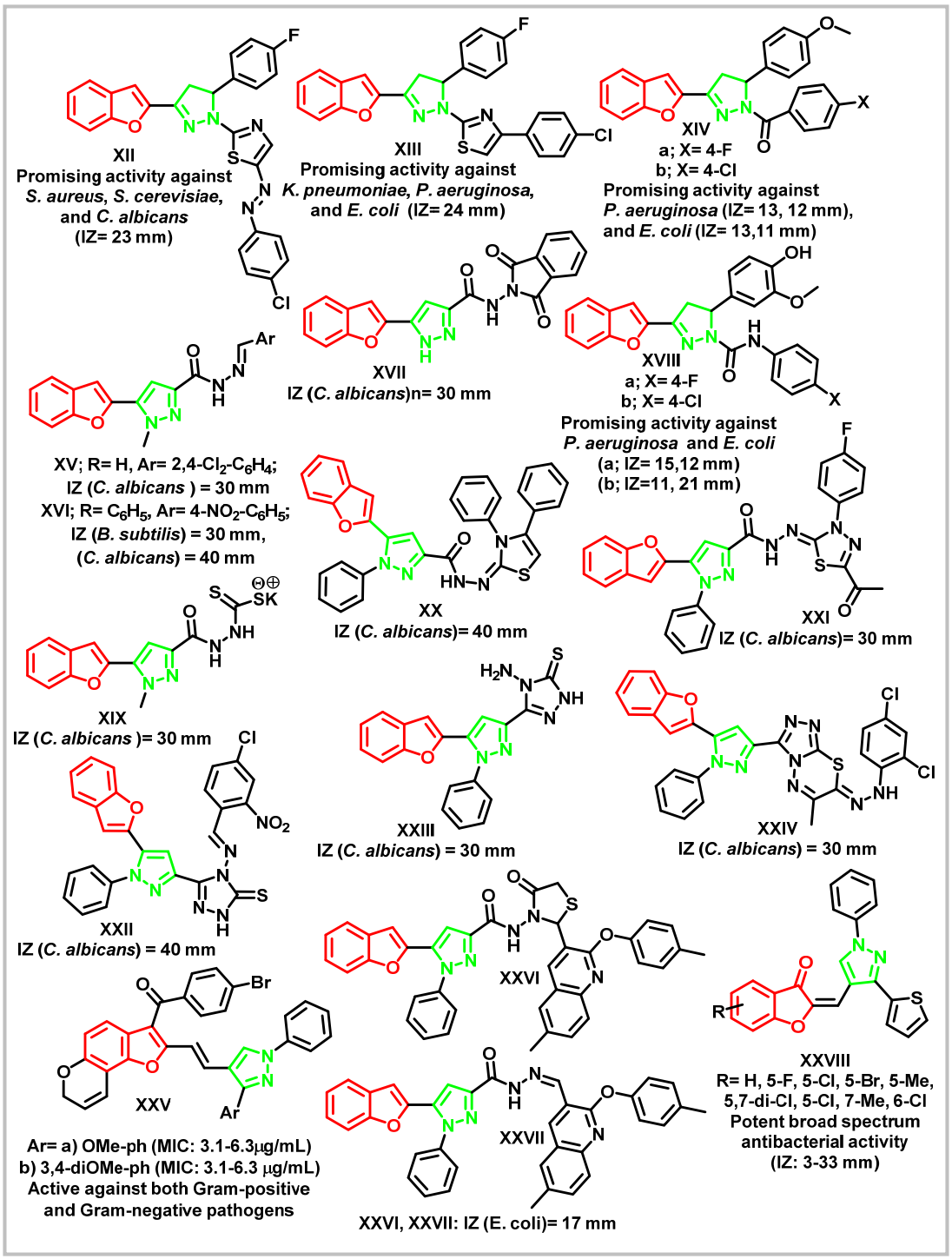

Figure 2. Examples of different molecules bearing benzofuran-pyrazole/pyrazoline scaffolds conjugated with heterocyclic rings possessing significant antimicrobial activities.

The topoisomerase enzyme is divided into three types in prokaryotic cells: type I, type II, and type III; in eukaryotes, type III is absent [41]. Type II has been divided into DNA gyrase (Gyrase A (GyrA) and B (GyrB), the two subunits of heterotetrameric DNA gyrase), and Topoisomerase IV [42]. Multiple studies have focused on Type II subunits due to their specificity in the operation of bacterial reproduction. The tyrosine residue in the GyrA subunit is considered the active site amino acid, which is important for releasing the twist in the DNA (negative supercoiling) as well as reunion (positive supercoiling), whereas the GyrB subunit has an active site that is responsible for ATPase potency, providing energy for the DNA supercoiling.

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DNA gyrase plays a unique function since it depends on negative supercoiling, a process that keeps balance in bacterial DNA replication and prevents positive supercoiling from over twisting DNA and breaking DNA strands. Accordingly, DNA gyrase has a bright future for the survival of bacteria. The numerous amounts of DNA gyrase in prokaryotes like bacteria and their absence in human cells make them a selective target for newly developed molecules preserving both antibacterial and anti-MDR efficacy [43]. On the other hand, different mutations in gyrase lead to bacterial resistance, which results in their traditional drug candidates not working properly. Based on these aspects, DNA gyrase inhibitors are driving the interest of researchers towards designing and synthesizing further potent candidates with higher DNA gyrase inhibitory activity and improving their toxicity profiles.

In order to produce molecules with increased bioactivity, a key tactic in drug discovery is molecular hybridization, which combines several bioactive pharmacophores into a single molecular entity. In addition, it has been acknowledged that nitrogen-containing heterocycles play a significant role as structural motifs in the creation of novel medications, especially in the area of antimicrobial agents [44,45]. Nitrogen and oxygen atoms produce a significant biological activity in heterocyclic structures by allowing them to interact with biological macromolecules like enzymes, proteins, and DNA [46–48].

Accordingly, herein we used the process of molecular hybridization to design and synthesize new hybrid molecules composed of benzofuran-pyrazole scaffold clubbed with various heterocycles, including pyridine, pyran, chromene, pyrano-pyrazole, pyrido-triazine and triazolo-pyridine nuclei **(Figure 3)**.

In total, a series of new hybrid compounds have been created, and their molecular structures have been fully characterized. The antimicrobial characteristics of these compounds were examined against various fungal isolates, Gram-positive and Gram-negative bacteria. Additionally, the new hybrids were evaluated as *in vitro* antioxidants and anti-inflammatory agents. The most promising compounds **9** and **10** were selected as a representative examples to study their suppression effects against *E. coli* DNA gyrase B enzyme. Molecular docking and ADMET studies were also performed for the most active compound **9** using *E. coli* DNA gyrase B as the target enzyme.

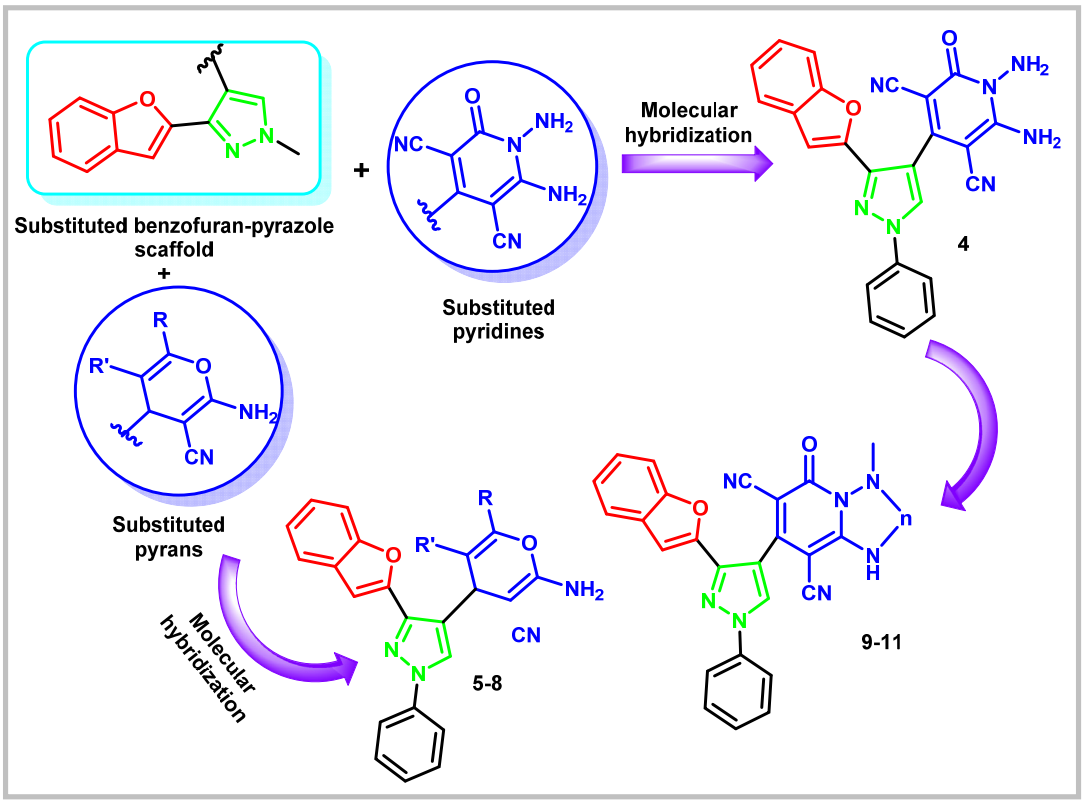

Figure 3. Development of new benzofuran-pyrazole hybrids of potential antimicrobial activity.

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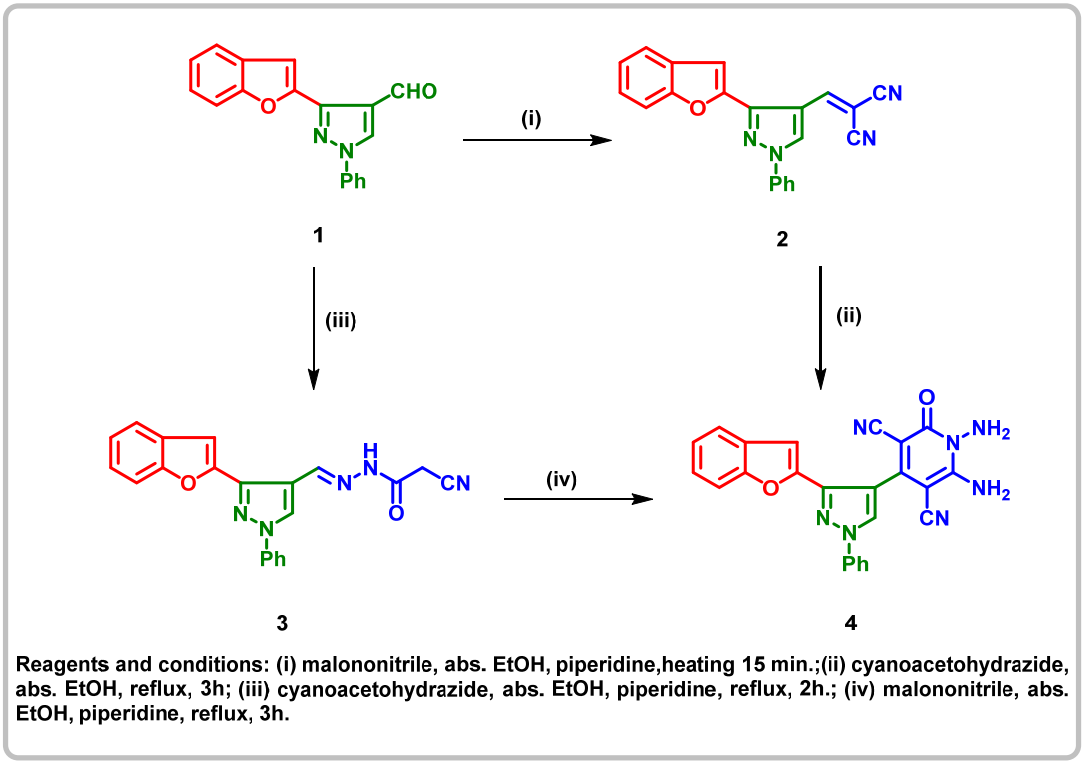
2. Results and Discussion

2.1. Chemistry

The synthetic pathways adopted for the preparation of the new benzofuranpyrazole hybrid derivatives in this study is depicted in (**Schemes 1-4**). Using Vilsmeier Haach reaction, the key starting material 3-(benzofuran-2-yl)-1-phenyl-1*H*pyrazole-4-carbaldehyde **(1)** was prepared according to the reported method [17]. Compound **1** was condensed with malononitrile to form 2-((3-(benzofuran-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene) malononitrile **(2)** [17]. The compound 1,6-diamino-4-(3-(benzofuran-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)-1,2-dihydro-2- oxopyridine-3,5-dicarbonitrile **(4)** was prepared by either two ways. Firstly, by refluxing the arylidine analogue **2** with cyanoacetohydrazide in absolute ethanol containing a catalytic amount of piperidine. Secondly, by condensation of the key starting aldehyde **1** with cyanoacetohydrazide to afford the cyanoacetohydrazide derivative **3** which was further cyclized with malononitrile in absolute ethanol containing few drops of piperidine to produce the desired compound **4**. The elemental analyses and spectral data confirmed the molecular structures of the synthesized derivatives. IR spectrum of compound **3** showed characteristic absorption bands at 3261, 2266 and 1678 cm^{-1} that are attributed to NH, CN and C=O groups, respectively. Also, its ^{1}H NMR spectrum showed the presence of four singlet signals at δ 4.24, 8.51, 8.58, 9.09 and 11.44 ppm referring to the methylene group, the azomethine CH=N (E and Z iomers), pyrazole-H5 and NH protons, respectively, in addition to the aromatic protons that appeared as multiplet signals at the range δ 7.31-8.05 ppm. Also, IR spectrum of the compound **4** displayed strong absorption bands at 3300, 3241, 3196, 3131 ($2NH_2$), 2218 (2CN) and at 1664 cm^{-1} (lactamic C=O). ^{1}H NMR spectrum of the same compound showed two D_2O exchangeable singlet signals at δ 5.72 and 8.58 ppm due to -C-NH_2 and -N-NH_2 protons, respectively. This result confirms the difference in the nucleophilicity between the two amino groups. Thus, it is expected that the hydrazide β-nitrogen (-N-NH_2) is more nucleophilic and will react more rapidly with the electron deficient carbon than the second amino group (-C-NH_2). The aromatic protons appeared as a multiplet signal at the range δ 7.17-8.01 ppm, while the pyrazole-H5 appeared as a singlet at δ 9.11 ppm. Compound **4** was further confirmed by its mass spectrum which agrees well with the assigned structure displaying the correct molecular ion peak at m/z 433 (**Scheme 1**).



Scheme 1. Synthesis of 1,6-diamino-2-oxopyridine-pyrazolobenzofuran derivative **4.**

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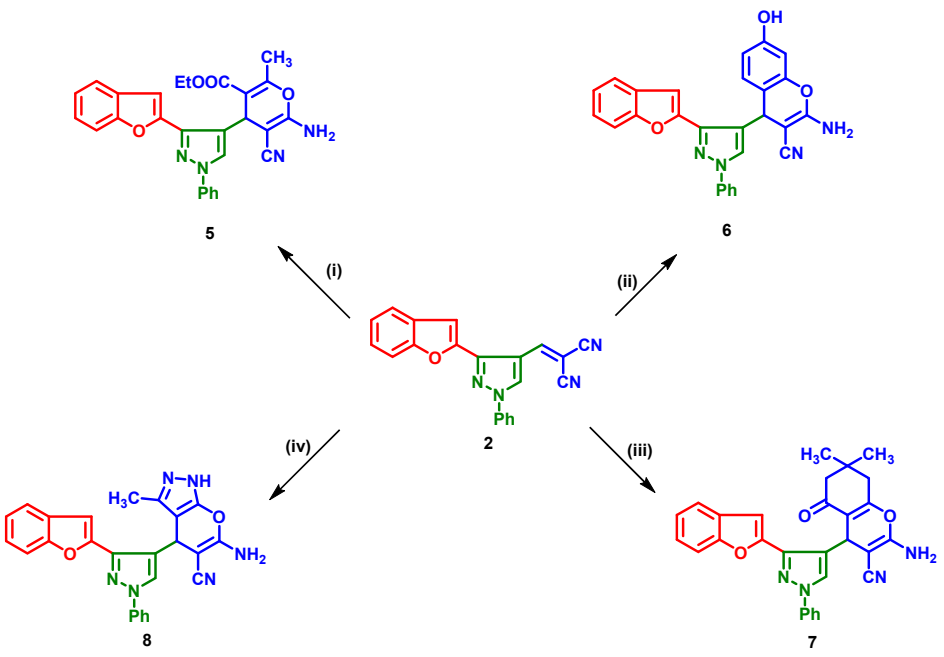
Moreover, condensation of the malononitrile derivative **2** with ethyl acetoacetate, in the presence of few drops piperidine as a catalyst yielded the corresponding ethyl 6-amino-5-cyano-2-methyl-4*H*-pyran-3-carboxylate **5**. The structure of the new pyran derivative was elucidated on the basis of elemental and spectral data. For example, ^{1}H NMR spectrum of compound **5** showed the characteristic triplet-quartet signals of the carboxylate group at δ 0.90 and 3.91 ppm, respectively. Also, four singlets appeared at δ 2.28, 4.45, 5.04, 7.71 ppm assignable to the protons of CH_3, NH_2, pyran-H4, and pyrazole-H5 respectively, besides to the multiplet signals of the aromatic protons that appeared at the range δ 7.17-7.70 ppm. Its ^{13}C NMR spectrum displayed three signals at δ 13.87, 18.58, 61.79 ppm related to the methyl and ethyl carbons, respectively, besides to the other signals attributed to the expected carbons of the molecule. Furthermore, the chromene derivatives **6**, **7** were furnished upon the treatment of the arylidine malononitrile derivative **2** with resorcinol and/or dimedone in absolute ethanol containing a catalytic amount of piperidine. The chemical structures of the obtained chromene and tetrahydrochromene derivatives **6**, **7** were confirmed by elemental and spectral analyses. For example, IR spectrum of compound **6** represented absorption bands at 3325, 3204, 3135, 2197 cm^{-1} contributed to OH, NH_2 and CN groups, respectively. ^{1}H NMR spectrum of the same analogue displayed three singlets at δ 5.21, 7.19, 8.61 ppm attributed to the pyran-H4, OH and pyrazole-H5, respectively. While ^{1}H NMR spectrum of compound **7** exhibited two singlet signals at δ 0.64 and 0.93 ppm refers to two methyl groups, Also, three singlet signals at 4.69, 6.99 and 8.53 ppm indicating the protons chromene-H4, NH_2 group and pyrazole-H5, respectively. In addition, ^{13}C NMR spectrum of **7** represented, besides the expected cyano and aromatic signals, characteristic signals at δ 26.59, 26.97, 28.86, 32.00, 50.52, 57.96, and 196.34 ppm due to the carbons of 2-CH_3, CH_2, chromene-C7, chromene-C6, chromene-C3, and C=O, respectively. Moreover, in order to obtain the fused pyrano[2,3-*c*]pyrazole compound **8**, the key intermediate **2** was treated with 3-methyl-1*H*-pyrazol-5(4*H*)-one derivative in absolute ethanol containing few drops of piperidine. The elemental and spectral data confirmed the chemical structure of compound **8**. IR spectrum of the latter compound showed characteristic absorption bands at the range 3393-3176 cm^{-1} due to NH, NH_2 and at 2186 cm^{-1} due to CN group. ^{1}H NMR spectrum exhibited a singlet signal at δ 1.83 ppm attributed to CH_3, besides the other signals that appeared at their expected regions. ^{13}C NMR spectrum represented a signal at δ 10.25 ppm assignable to CH_3 group, in addition to other 24 signals representing the cyano and aromatic carbons of the molecule (**Scheme 2**).



Reagents and conditions: (i) ethyl acetoacetate, abs. EtOH, piperidine, stirring overnight; (ii) resorcinol, abs. EtOH, piperidine, reflux, 1h; (iii) dimedone, abs. EtOH, piperidine, stirring overnight; (iv) 3-methyl-1*H*-pyrazol-5(4H)-one, abs. EtOH, piperidine, stirring overnight.

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Scheme 2. Synthesis of pyrano, chromene, tetrahydrochromene, pyrano[2,3-*c*]pyrazole derivatives.

O-Diamines are ready-made nucleophilic centers for the synthesis of fused nitrogen heterocyclic rings. Thus, diaminopyridone **4** was a useful building block for the synthesis of nitrogen bridge-head pyrido-triazine and/ or pyrido-triazole derivatives **9**, **10**, **11a-d**, respectively. Upon the treatment of diaminopyridone **4** with 1,2- dibromoethane in pyridine produced the corresponding tetrahydro-1*H*-pyrido[1,2-*b*][1,2,4]triazine derivative **9**. Elemental analysis and spectral data (IR, MS, ^{1}H and ^{13}C NMR spectra) confirmed the reaction product. IR spectrum showed absorption bands at 3189, 3127, 2213 cm^{-1} due to 2NH and 2CN groups, respectively. In addition, its ^{1}H NMR spectrum displayed the methylene protons of 2CH_2 as two multiplets at δ 2.07, 2.43 ppm, in addition to the aromatic protons, pyrazole-H5, 2NH protons that appeared at their expected regions. Furthermore, ^{13}C NMR spectrum of the same compound exhibited 25 carbon signals, the most important signals appeared at δ 31.12, 71.45, 161.64 ppm characteristic for the two methylene and carbonyl carbons, respectively. Furthermore, the 1,2,4-triazolo[1,5-*a*]pyridine and tetrahydro-[1,2,4]-triazolo[1,5-*a*]pyridine derivatives **10, 11a-d** were obtained by boiling of the diaminopyridone derivative **4** in acetic anhydride and/or its refluxing with different aldehydes in acetic acid, respectively. IR spectrum of compound **10** showed the disappearance of NH_2 bands of the parent diaminopyridone **4** and the presence of two absorption bands at 2223, 1671 cm^{-1} referring to 2CN, and 2CO groups, respectively. Also, ^{1}H NMR spectrum of compound **10** revealed, in addition to the aromatic protons and pyrazole-H5, two characteristic singlet signals at δ 1.92, 2.09 ppm attributed to the methyl and acetyl protons, respectively. While its ^{13}C NMR spectrum displayed 24 carbon signals, representing the methyl and acetyl carbons at δ 21.56 and 31.19 ppm, respectively. At the same time, the elemental analyses and spectral data were in consistent with the proposed tetrahydro-[1,2,4]-triazolo[1,5-*a*]pyridine derivatives **11a-d**. For example, ^{1}H NMR spectrum of compound **11a** revealed two singlets at δ 3.79, 3.89 ppm assignable 3(OCH_3), three other singlet signals at δ 8.56, 8.90, 9.13 ppm attributable to the respective protons of 2NH, triazole-H3 and pyrazole-H5 respectively. Furthermore, ^{1}H NMR spectrum of **11c** exhibited, besides the expected signals of the aromatic protons, four singlets at δ 2.40, 8.70, 9.07, 9.12 ppm due to the protons of CH_3, 2NH and pyrazole-H5, respectively. In addition, the furan-H3, H4 appeared as two douplets at δ 6.51, 6.96 ppm, respectively. Mass spectra of **11a-d** showed the molecular ion peaks which were in agreement with their molecular formulae (**Scheme 3**).

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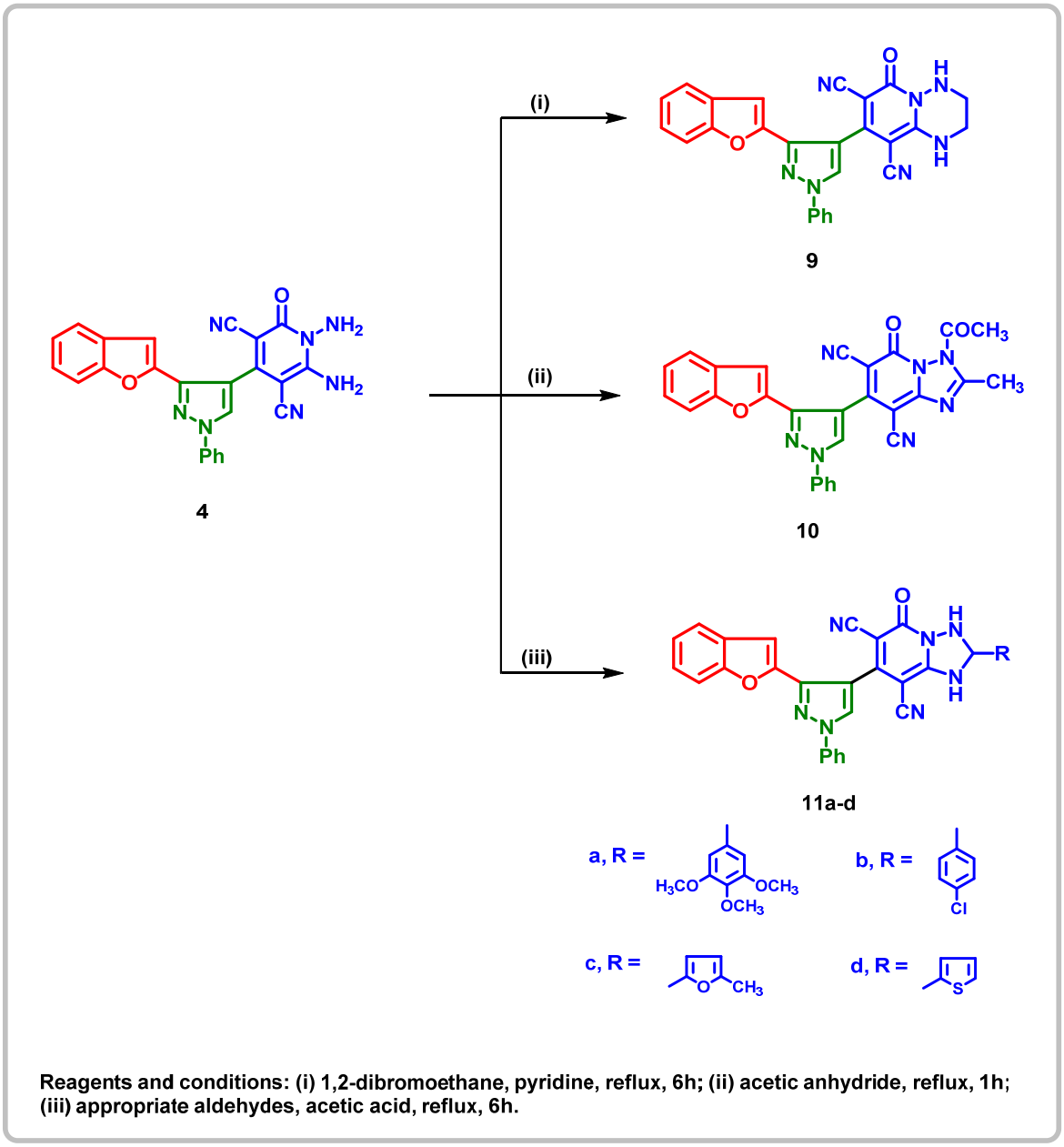

Scheme 3. Synthesis of pyrido[1,2-*b*][1,2,4]triazine and [1,2,4]triazolo[1,5-*a*]pyridine derivatives.

2.2. Biological Evaluation

2.2.1. Antimicrobial Activity Determination

The *in vitro* antimicrobial activity of the new target compounds was evaluated against two fungal isolates, *F. solani* and *C. albicans* ATCC-10231; four bacterial isolates, *S. aureus* ATCC 6538 and *B. cereus* ATCC 11778, as Gram-positive bacteria; and *E. coli* 25922 and *P. aeruginosa* ATCC 27853 as Gram-negative bacteria. These specific strains were chosen because of their ability to form biofilms and their major impact on human health. Using the agar-well diffusion method [49], the average diameter of the inhibition zones in millimetres was assessed for each tested compound (10 µg/mL) against each type of microbial growth surrounding the discs **(Table 1)**.

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Table 1. The antimicrobial potency of the new target benzofuran-pyrazole based derivatives, expressed as inhibition zone (mm).

Mean diameter of zones of inhibition (Mean ± SEM) (mm)						
Compd. No.	Gram +ve Bacteria		Gram -ve bacteria		Fungi	
	S. aureus ATCC 6538	***B. cereus ATCC-11778***	***E. coli ATCC-25922***	***P. aeruginosa ATCC-27853***	***F. solani***	***C. albicans ATCC-10231***
2	15	13	18	20	12	12
4	15	15	15	17	17	15
5	15	12	14	20	12	11
6	14	12	15	17	0	0
7	15	14	15	15	0	0
8	20	16	15	17	16	15
9	**20**	**18**	**15**	**20**	**16**	**20**
10	**22**	**19**	**15**	**20**	**20**	**20**
11a	15	12	15	18	15	0
11b	**20**	15	15	20	15	16
11c	20	15	17	20	12	15
11d	**20**	15	16	17	15	19
N*	20	20	25	24	-	-
C*	-	-	-		20	14

Antibacterial Standard; N*= Novobiocin (30 µg), antifungal Standard; C*= Clotrimazole (50 µg).

Furthermore, minimum inhibitory concentration (MIC) values (mentioned in µM) for the promising compounds exhibiting a IZ ≥ 15 µM (**4**, **7**, **8**, **9**, **10**, **11b**, **11c**, **11d**) were detected using the double-sequence dilution method [50–52]. Novobiocin (30 µg) and clotrimazole (50 µg) were utilized as the standard antibiotic and antifungal drugs, respectively, and the results were recorded in **Table 2**.

Table 2. MIC values of the most active analogues against various microbial species **(µM).**

Compd. No.	Gram +ve Bacteria		Gram -ve bacteria		Fungi	
	S. aureus ATCC 6538	***B. cereus ATCC-11778***	***E. coli ATCC-25922***	***P. aeruginosa ATCC-27853***	***F. solani***	***C. albicans ATCC-10231***
4	15	15	15	17	27	25
7	15	14	15	15	**27**	**30**
8	20	16	15	17	35	25
9	**2.50**	**4.65**	**5.79**	**17.60**	**20**	**16**
10	**3.49**	**8.80**	**4.65**	**20**	**16**	**14**
11b	**5.11**	**8.60**	**8.69**	**17.0**	**15**	**16**
11c	**5.90**	**7.79**	**16.0**	**16.90**	**15**	**19**
11d	**5.11**	**15**	**16.0**	**20**	**15**	**19**
N*	3.49	6.98	4.65	18.6	-	-
C*	-	-	-		20	14

Antibacterial Standard; N*= Novobiocin (30 µg), antifungal Standard; C*= Clotrimazole (50 µg).

Based on MIC results, it was noticed that all of the tested strains were susceptible to the antibacterial characteristics of the examined compounds. It was detected that the nitrogen bridge-head pyrido-triazine and/or pyrido-triazole derivatives **9**, **10**, and **11b-d**, respectively, were the most

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promising candidates as broad-spectrum antimicrobial members. While it showed an equivalent activity to clotrimazole against the examined fungal strains (MIC = 20, 16 µg/mL, $MIC_{Clotrimazole}$= 20, 14 µg/mL), the six-membered pyrido-triazine compound **9** produced more significant potency than novobiocin against the tested positive and negative bacterial isolates (MIC= 2.50 -17.60 µg/mL, $MIC_{novobiocin}$= 3.49-18.6 µg/mL). On the other hand, the 2-methyl-triazolo[1,5-*a*]pyridine derivative **10** has emerged as a potent antifungal candidate. It showed more significant activity against the fungal strain *F. solani* than clotrimazole, with an MIC value of 16 µg/mL, and equivalent antifungal activity to the reference drug against the tested *C. albicans* isolates, with an MIC value of 14 µg/mL. The antibacterial efficacy of the latter derivative appears to be slightly lower than that of the reference novobiocin, with MIC values ranging from 3.49 to 20 µg/mL.

Interestingly, the replacement of the 2-methyl group of compound **10** with different substituted phenyl groups as compounds **11b-c** enhanced the antifungal activity more than that produced by clotrimazole, with MIC values ranging from 16–19 µg/mL, but it reduced the antibacterial activity against the examined bacterial isolates, producing MIC values less than those produced by novobiocin, ranging from 5.11–20 µg/mL. Conversely, the rest of the target compounds (**4**, **7**, **8**) exhibited moderate antimicrobial activity with MIC values ranging from 14 to 30 µM. Taken together, it could be concluded that the pyrido-triazine and/or pyrido-triazole ring system attached to the pyrazol-benzofuran scaffold is a highly recommended feature for gaining significant broad spectrum antimicrobial efficacy.

2.2.2. Antioxidant Activity

Free radicals display an integral role in normal physiological functions such as cell signaling, immunological response, and overall redox homeostasis maintenance [53]. Reactive Oxygen and Nitrogen Species (ROS/RNS) are naturally produced through the cellular metabolism. They act as so-called "redox messengers" in signalling pathways and are helpful against infectious pathogens, but they can also cause detrimental oxidative stress [53,54]. The relationship between endogenous and exogenous antioxidant systems regulates the level of radical activity. Excess radicals can cause severe oxidation and damage to a variety of biomolecules, which can result in dysfunction or even cell death. Numerous neurological diseases, cancer, diabetes, high blood pressure issues, and cardiovascular diseases, are believed to be associated with free radical damage [55]. Antioxidants are compounds that reduce free radical concentrations, thus act as a protective barrier against the damage they cause to the body, which is why they are crucial in the prevention of various diseases.

Many processes have been described to evaluate the antioxidant activity of specific compounds, but the most widely documented relates to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [56] using the DPPH-free radical scavenging assay utilizing ascorbic acid as a standard drug and results were presented in a **Table 3**. The obtained antioxidant activity of the tested compounds varied from moderate to potent activity in comparison with ascorbic acid. The most potent scavenging activity was produced by the compounds **4**, **6**, **9**, **11b**, **11d** (%DPPH scavenging activity = 84.16, 86.42, 85.87, 90.52, 88.56, 89.42%, respectively). On the other hand, the rest of the derivatives appeared to be moderate to weak antioxidant candidates **(Figure 4)**. Fortunately, the most potent antimicrobial pyrido-triazine derivative **9** produced an effective radical scavenging capacity (88.56±0.43%).

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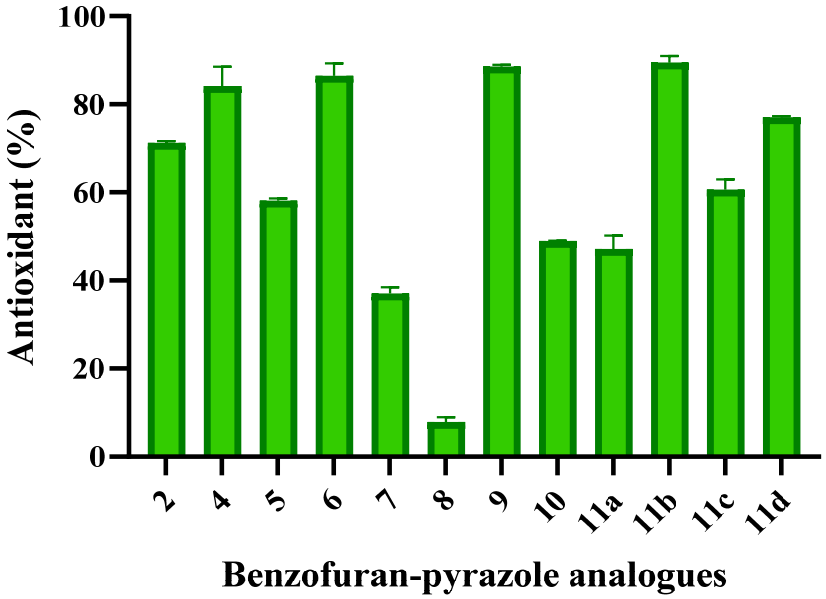

Figure 4. Antioxidant activities of the new target benzofuran-pyrazole based derivatives.

2.2.3. Anti-Inflammatory Assay

The new target compounds were assessed for their *in vitro* anti-inflammatory potential via the HRBC membrane stabilization process. The anti-inflammatory effectiveness of the compounds was determined by regulating the suppression of hypotonicity-induced HRBC membrane lysis, which is an inflammatory response.

The HRBC membrane can be viewed as a model of the lysosomal membrane, which is crucial in inflammation. Stabilization of the lysosomal membrane plays a key role in controlling inflammatory reactions [57]. The mechanism involves the release of lysosomal contents of active neutrophils, such as bactericidal enzymes and proteases, which, upon extracellular release, produce further tissue inflammation and injury which is said to be acute or chronic inflammation [57].

The HRBC membrane stability test relies on the discovery that non-steroidal anti-inflammatory drugs prevent erythrocyte lysis, most likely by maintaining the cell membrane's stability. Inflammatory symptoms may be alleviated by compounds that stop the lysis of the HRBC membrane brought on by the release of hydrolytic enzymes from lysosomes [58]

The mechanism of action for compounds' membrane protection may involve their binding to HRBC membranes and altering the charges on the cell surface [59,60]. Alternatively, it may involve the deformation of cells through interactions with other compounds or membrane proteins in the erythrocyte membranes. Later on, this contact can cause changes to the cell surface charges (Oyedapo et al., 2004)or their ability to adjust the intracellular concentration of calcium into the erythrocytes [61]. Accordingly, it was of interest to predict the anti-inflammatory activity *in vitro* of the new analogues according to the reported method [62].

The percentage of HRBC membrane stabilization test for the newly synthesized derivatives and the positive control aspirin and diclofenac sodium were carried out and the results were provided in **Table 3**.

Most of the examined derivatives showed significant HRBC membrane stabilization and protection percentages ranging from 86.70±0.259 to 99.25±0.108%. Unfortunately, less protection % and weak activity were determined by compounds **11c** and **10** (73.67±0.388 and 29.67±0.496%, respectively). Interestingly, the most active antimicrobial analogue **9** showed promising protection % (86.70±0.259%) **(Figure 5)**

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Table 3. *In vitro* anti-inflammatory and antioxidant activities of the new target benzofuran-pyrazole based derivatives.

Compound No.	Anti-inflammatory		Antioxidant activity
	% Hemolysis	**% Protection**	**%DPPH radical scavenging activity**
2	0.82±0.108	99.25±0.108	71.19±0.43
4	0.67±0.194	99.19±0.194	84.16±4.41
5	0.76±0.151	99.13±0.151	58.10±0.52
6	0.76±0.086	99.18±0.086	86.42±2.85
7	2.38±0.173	97.50±0.173	37.06±1.38
8	1.43±0.173	98.44±0.173	7.89±1.04
9	**13.12±0.259**	**86.70±0.259**	**88.56±0.43**
10	70.68±0.496	29.67±0.496	48.93±0.09
11a	1.37±.086	98.57±0.086	47.16±3.03
11b	6.16±0.755	93.30±0.755	89.42±1.56
11c	26.60±0.388	73.67±0.388	60.61±2.34
11d	2.84±0.259	97.35±0.259	77.00±0.26
Aspirin	1.98±0.173	97.90±0.173	----
Diclofenac	0.46±0.043	99.51±0.043	-----
Ascorbic acid	----	----	100±0.00

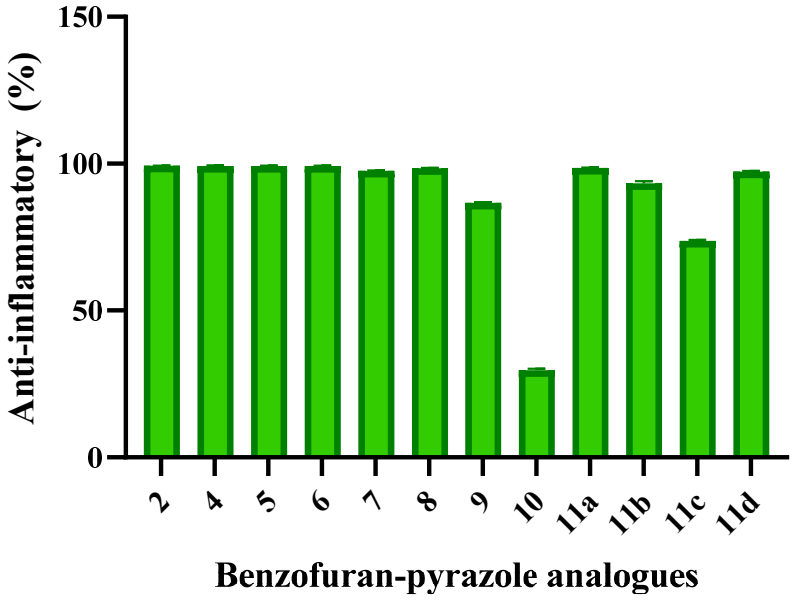

Figure 5. Anti-inflammatory activity of the new target benzofuran-pyrazole-based derivatives.

2.2.4. E. coli DNA Gyrase B Suppression Effect

The compounds that exhibited the most promising antimicrobial, antioxidant, and anti-inflammatory activities, **9** and **10** were evaluated for *in vitro* suppression effects against *E. coli* DNA gyrase B as a trial to find out their modes of action as antimicrobial candidates [62–64], where novobiocin was served as a standard drug **(Table 4)**.

The results demonstrated that the pyrido-triazine compound **9** produced a more potent suppression impact against *E. coli* DNA gyrase B than **10**, but was about threefold less potent than novobiocin, with IC_{50}s of 10.71±0.21, 19.58±0.03, and 3.64 ± 0.10, respectively. The increase in the ring

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size attached to the benzofuran-pyrazole mother scaffold led to a greater suppression effect, which could be explained by the creation of an additional hydrophobic interaction with *E. coli* DNA gyrase B, resulting in an increase in the fitting of the compound in the active sites of the tested enzyme.

3.4. In Vitro Cytotoxicity Assay

In continuation of this study, the safety profiles of the analogues **9**, and **10** were assessed utilizing the calorimetric cell proliferation MTT [64] to evaluate the cytotoxic effects against the human diploid cell line WI-38, which is derived from lung tissues. The safety profiles of the compounds exhibited promise and are significantly superior to those of the reference drug novobiocin (IC_{50}s = 163.3± 0.17, 170 ± 0.40, and 86.2± 0.03 µM, respectively), as shown in **Table 4**.

Table 4. Assessment of inhibitory potential of compounds **9**, **10** against *E. coli* DNA gyrase B in relation to novobiocin.

Compd. No.	IC_{50} (mean±SEM) (µM)	
	E. coli DNA gyrase B	Cytotoxicity WI38
9	**10.71±0.21**	163.3± 0.17
10	**19.58±0.03**	170 ± 0.40
Novobiocin	3.64 ± 0.10	86.2± 0.03

2.3. In Silico Studies

2.3.1. Molecular Docking

Despite docking software's capacity to identify potential binding modes between a ligand and its target, it is still an unreliable method that requires constant verification. As a result, the docking procedure was initially verified by re-docking the co-crystallized ligand close to the enzyme's binding site. For the co-crystallized ligand of the DNA gyrase enzyme, the root mean square deviation (RMSD) was found to be 0.23 indicating a successful docking technique (**Figure 6**).

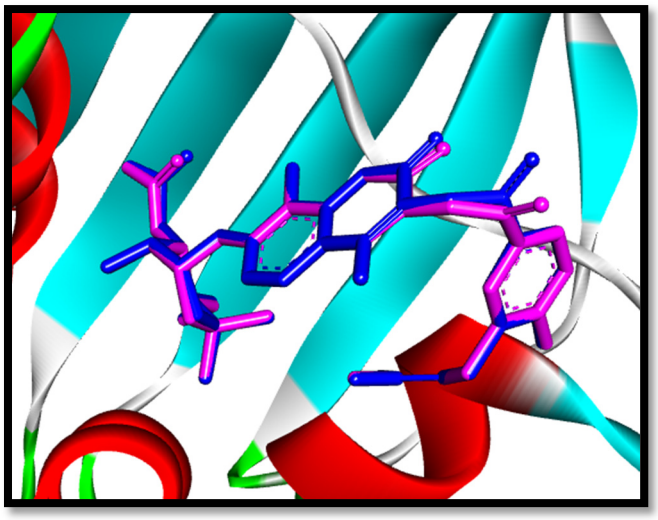

Figure 6. 3D representation of the superimposition of the co-crystallized (purple) and the docking pose (blue) of novobiocin in the active site of *E. coli* DNA gyrase.

Compound **9** has the ability to bind to DNA gyrase active site in a comparable pattern to the co-crystallized ligand; novobiocin. It shows a binding energy score of -8.9 kcal/mol. compared to -9.4 kcal/mol. for novobiocin. Compound **9** binds the key amino acids Arg144, Asp81, Arg84 and Glu58

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through pi-anion and pi-cation interactions in addition to supportive hydrophobic interactions with different amino acid residues in the vicinity of the active site **(Figure 7)**.

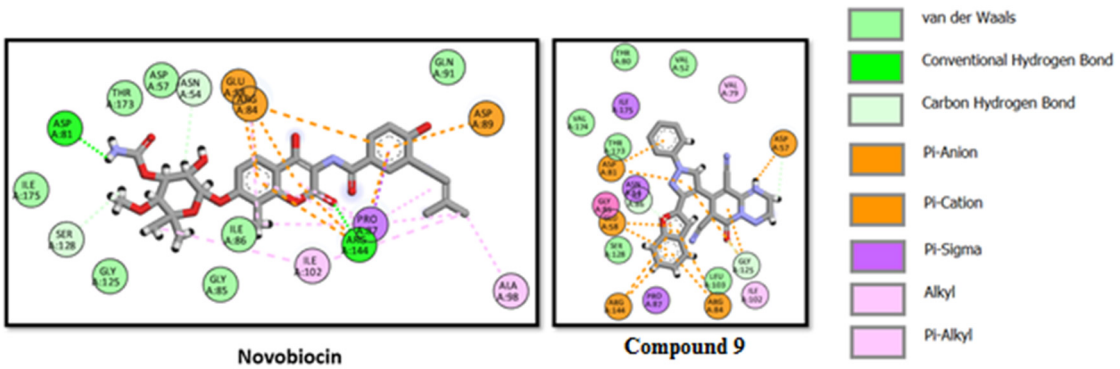

Figure 7. The binding interactions of novobiocin against the tested compound **9** against *E. coli* DNA gyrase active site (pdb: 4URO).

2.3.2. ADMET Study of Compound 9

Several measures are calculated by the SwissADME webserver to predict its oral bioavailability, pharmacokinetic properties, BBB penetration possibility, and permeability glycoprotein (PGP) binding affinity. The bioavailability radar chart of compound **9** shows only one violation out of six measured parameters: lipophilicity (LIPO), size, polarity, insolubility (INSOL), insaturation (INSATU), and flexibility (FLEX); the violation was in the insaturation parameter (Figure 9). A BOILED-EGG chart was constructed; the white area indicates gastro-intestinal absorption, while the non-mutually exclusive yellow area indicates BBB penetration. The blue color indicates the high possibility of the tested compound being a substrate for PGP, which is an efflux protein responsible for uptake and efflux of many drugs; a red color means the lower possibility of being a PGP substrate [67].

Compound **9** shows good gastro-intestinal absorption with no BBB penetration, but it's a possible substrate for PGP (Figure 10). Eventually, applying Lipinski's rule of five [68] which is a four-rule fulfilment criteria that is calculated to predict the drug-likeness of the tested derivative, 11 has a predicted logP value of 2.67 (<5), a molecular weight of 459.46 g/mol (<500), 9 hydrogen bond acceptor groups (7 nitrogens + 2 oxygens) (<10), and only 2 hydrogen bond donor groups (2 NH) (<5), and so shows no violation of Lipinski's rule, indicating its good pharmacokinetic profile.

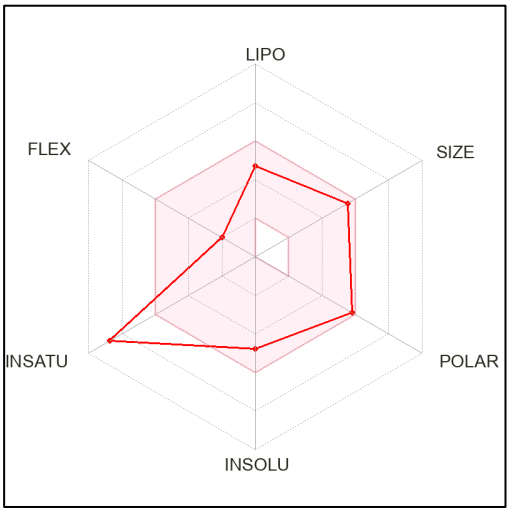

Figure 9. Bioavailability radar chart for compound **9** (The pink area indicates the accepted range for each of the measured parameter).

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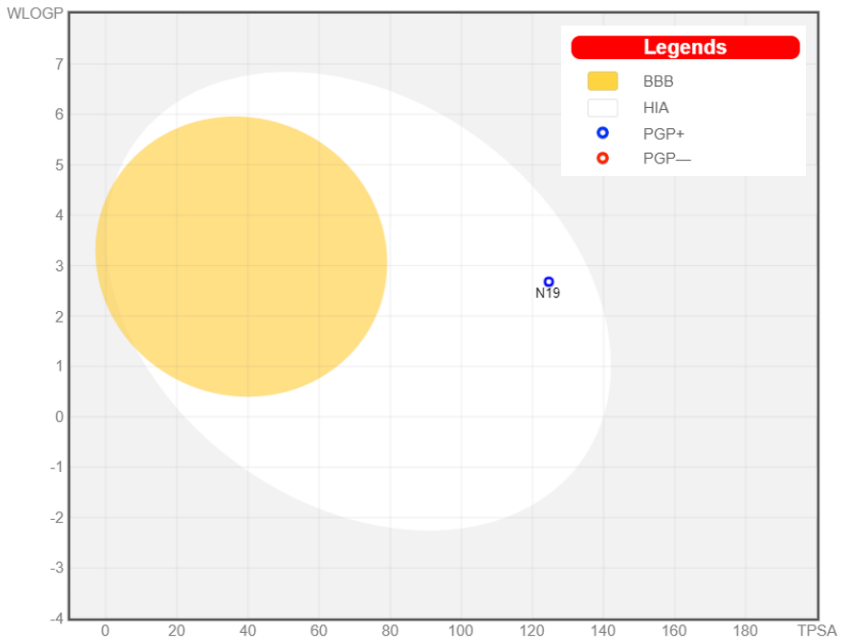

Figure 10. BOILED-EGG chart for compound **9** (the blue circle represents the tested compound).

4. Conclusion

Based on the molecular hybridization process, new hybrid molecules composed of benzofuran-pyrazole scaffolds are conjugated with different *N*/*O* heterocyclic rings, such as pyridine, pyran, chromene, pyrano-pyrazole, pyrido-triazine, and triazolo-pyridine cores. The newly synthesized target compounds were characterized using microanalysis and spectroscopic approaches. In addition, the new analogues were evaluated as antimicrobial candidates against various strains of both Gram-positive and Gram-negative bacteria as well as fungi in comparison with novobiocin and clotrimazole as antibacterial and antifungal standard drugs. Promising broad-spectrum antimicrobial potency against the examined bacterial and fungal species was observed by the pyrido-triazine and/or pyrido-triazole derivatives **9**, **10**, and **11b-d**, respectively, with MIC values ranging from 2.50–20 µg/mL. All the new hybrids were also examined as *in vitro* antioxidants and anti-inflammatory agents using DPPH-free radical scavenging assay and HRBC membrane stabilization processes, respectively. Compounds **4**, **6**, **9**, **11b**, **11d** exhibited the most potent scavenging activity (%DPPH scavenging activity = 84.16 - 90.52%), while all of the examined compounds (except **11c** and **10**) showed significant HRBC membrane stabilization and protection percentages ranging from 86.70±0.259 to 99.25±0.108%. Moreover, the most promising antimicrobial, antioxidant, and anti-inflammatory activities, **9** and **10** were evaluated for *in vitro* suppression effects against *E. coli* DNA gyrase B to find out their expected modes of action as antimicrobial candidates. The compound **9** was a more potent inhibitor against DNA gyrase B than **10**, with IC_{50}s of 10.71 and 19.58, respectively. Both **9** and **10** revealed promising safety profiles against the normal human diploid cell line WI-38 cell line, which were significantly superior to that obtained by the reference drug novobiocin (IC_{50}s = 140 ± 0.36, 165 ± 0.40, 163.3 ± 0.17, and 86.2 ± 0.09 µM, respectively).

The hero candidate in this study was the pyrido-triazine derivative **9**, which exhibited significant broad-spectrum antimicrobial activity with a radical scavenging capacity of 88.56±0.43%, a hemolytic protection rate of 86.70±0.259%, and *an E. coli* DNA gyrase B suppression impact with promising safety profile. So, it could be considered a basic scaffold for the further development of new antimicrobial agents that aid in overcoming the drug resistance phenomenon.

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5. Experimental Protocols

5.1. Chemistry

All melting points are uncorrected and were taken in open capillary tubes using Electrothermal apparatus 9100. The instruments used to determine melting points, spectral data (IR, ^{1}H NMR, ^{13}C NMR, and mass), as well as chemical analyses were included in a detailed description of the in the file of Supporting Information.

3-(Benzofuran-2-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde **(1)** and 2-((3-(benzofuran-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)malononitrile **(2)** were prepared according the reported method [17].

6.1.1. N′-((3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)methylene)-2-cyanoacetohydrazide (3)

A mixture of 3-(benzofuran-2-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (**1**) (2.88 g, 0.01 mol) and cyanoacetohydrazide (0.99 g, 0.01 mol) in absolute ethanol (30 mL) was refluxed for 3h. Upon cooling, the formed precipitate was filtered, dried and recrystallized from ethanol to give the title compound **3**.

Yield 61%, mp. 211-213^{o}C, yellow powder; IR (KBr, cm-1): 3261 (NH), 3059 (CH-arom.), 2959, 2917 (CH-aliph.), 2267 (CN), 1678 (C=O, amide); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 3.86 (s, 2H, CH_2, Z-isomer), 4.24 (s, 2H, CH_2, E-isomer), 7.31-7.46 (4H, m, Ar-H), 7.57-7.76 (6H, m, Ar-H), 7.89-8.05 (3H, m, Ar-H), 8.51 (s,1H, CH=N, E-isomer), 8.58 (s,1H, CH=N, Z-isomer), 9.09 (s, 1H, pyrazole-H5), 11.44 (s, 1H, NH, D_2O exchangeable); MS, m/z (%): 369 [M^{+}] (12), 285 [$C_{18}H_{11}N_3O$] (8), 77 [C_6H_5] (100); Anal. For $C_{21}H_{15}N_5O_2$ (369.38): Calcd. C, 68.28; H, 4.09; N, 18.96; Found: C, 68.34; H, 4.24; N, 18.71.

1,6-Diamino-4-(3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-1,2-dihydro-2-oxopyridine-3,5-dicarbonitrile (4)

Method (A):

A mixture of 2-((3-(benzofuran-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)malononitrile (**2**) (3.36 g, 0.01 mol) and cyanoacetohydrazide (0.99 g, 0.01 mol) in absolute ethanol (30 mL) containing 3-5 drops of piperidine was refluxed for 2h. The white precipitate obtained during heating was filtered, dried and recrystallized from ethanol to give the title compound **4**.

Method (B):

A mixture of compound **3** (1.85 g, 0.005 mol) and malononitrile (0.33 g, 0.005 mol) in absolute ethanol (20 mL) containing 3-5 drops of piperidine was refluxed for 3h. The white precipitate obtained during heating was filtered, dried and recrystallized from ethanol to give the title compound **4**.

Yield 85% (A), 80% (B); mp 269-270 ^{o}C; IR (ν_{max}/cm^{-1}): 3393, 3315, 3210 (2NH_2), 2217 (CN), 1661 (CO); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 5.72 (s, 2H, NH_2, D_2O exchangeable), 7.17 (s, 1H, Ar-H), 7.27-7.47 (m, 3H, Ar-H), 7.59-7.70 (m, 4H, Ar-H), 8.01 (d, 2H, ^{3}J = 7.8 Hz, Ar-H), 8.58 (br, 2H, NH_2, D_2O exchangeable), 9.11 (s, 1H, pyrazole proton) ppm; ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 75.998, 88.179, 105.392, 111.812, 115.796, 115.875, 116.718, 119.176, 122.06, 123.908, 125.691, 128.043, 128.631, 130.404, 139.112, 141.566, 148.516, 151.384, 154.606, 157.174, 159.736. MS, m/z (%): 433 [M^{+}] (37), 434 [M^{+1}] (19), 77 [due to C_6H_5] (100). Anal. calcd. for $C_{24}H_{15}N_7O_2$ (433.43): C, 66.51; H, 3.49; N, 22.62; found: C, 66.32; H, 3.22; N, 22.43.

6.1.3. Ethyl 6-amino-4-(3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-5-cyano-2-methyl-4H-pyran-3-carboxylate (5)

A mixture of compound **2** (0.68 g, 0.002 mol) and ethyl acetoacetate (0.26 ml, 0.002 mol) in absolute ethanol (20 mL) containing 2-3 drops of piperidine was stirred overnight at room temperature. The formed precipitate was filtered, dried and recrystallized from ethanol to give the title compound **5**.

Yield 76%, mp. 196-198^{o}C, white powder; IR (KBr, cm^{-1}): 3315, 3188, (NH_2), 3062 (CH-arom.), 2980, 2924 (CH-aliph.), 2201 (CN), 1725 (C=O, ester); ^{1}H NMR (300 MHz; $CDCl_3$) δ_H 0.90 (t, 3H, ^{3}J = 7.2 Hz, CO2CH2*CH3*), 2.28 (s, 3H, CH_3), 3.91 (q, 2H, ^{3}J = 6.8 Hz, CO2*CH2*CH3), 4.45 (s, 2H, NH_2, D_2O exchangeable), 5.04 (s,1H, pyran-H4), 7.17-7.29 (m, 4H, Ar-H), 7.39-7.50 (m, 3H, Ar-H), 7.56-7.59 (m,

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1H, Ar-H), 7.67-7.70 (m, 2H, Ar-H), 7.71 (1H, s, pyrazole-H5); ^{13}C NMR (75 MHz; $CDCl_3$) δ_C 13.869 (CH_3), 18.880 (CH_3), 29.076 (pyran-C-4), 60.872 (pyran-C-5), 61.788 (CH_2), 104.257, 107.649, 111.333, 119.024, 119.387, 121.316, 123.076, 124.410, 125.764, 127.023, 127.896, 128.755, 129.551, 139.759, 142.550, 150.362, 155.004, 156.562, 157.613 (aromatic-C), 166.016 (CO); MS, m/z (%): 466 [M^+] (3), 465 [M^+-1] (3), 77 [C_6H_5] (100); Anal. For $C_{27}H_{22}N_4O_4$ (466.49): Calcd. C, 69.52; H, 4.75; N, 12.01; Found: C, 69.81; H, 4.93; N, 12.21.

2-Amino-4-(3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-7-hydroxy-4H-chromene-3-carbonitrile (6)

A mixture of compound **2** (0.68 g, 0.002 mol) and resorcinol (0.22 g, 0.002 mol) in absolute ethanol (20 mL) containing 2-3 drops of piperidine was refluxed for 1h. The formed precipitate during heating was filtered, dried and recrystallized from ethanol to give the title compound **6**.

Yield 57%, mp. 252-254oC, yellow powder; IR (KBr, cm^{-1}): 3325, 3204, 3135 (OH, NH_2), 3060 (CH-arom.), 2197 (CN); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 5.21 (s, 1H, pyran-H4), 6.41-6.46 (m, 2H, Ar-H), 6.83-6.87 (m, 3H, Ar-H), 7.19 (s, 1H, OH, D_2O exchangeable), 7.24-7.36 (m, 4H, NH_2, D2O exchangeable, Ar-H), 7.54 (t, 2H, ^3J = 8.1 Hz, Ar-H), 7.63 (t, 2H, ^3J = 8.7 Hz, Ar-H), 7.95 (d, 2H, ^3J = 7.5 Hz, Ar-H), 8.61 (1H, s, pyrazole-H5); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 30.726 (Chromene-C4), 55.838 (Chromene-C3), 102.607, 103.898, 111.714, 112.667, 113.299, 118.741, 121.412, 121.721, 123.753, 125.059, 127.209, 127.386, 128.575, 129.434, 130.091, 130.159, 139.555, 141.735, 149.439, 150.498, 154.559, 157.598, 160.696 (aromatic-C); MS, m/z (%): 446 [M^+] (5), 429 [M^+-OH] (42), 77 [C_6H_5] (100); Anal. For $C_{27}H_{18}N_4O_3$ (446.46): Calcd. C, 72.64; H, 4.06; N, 12.55; Found: C, 72.92; H, 4.27; N, 12.26.

2-Amino-4-(3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-5,6,7,8-tetrahydro-7,7-dimethyl-5-oxo-4H-chromene-3-carbonitrile (7)

A mixture of compound **2** (0.68 g, 0.002 mol) and dimedone (0.28 g, 0.002 mol) in absolute ethanol (20 mL) containing 2-3 drops of piperidine was stirred overnight at room temperature. The formed precipitate was filtered, dried and recrystallized from ethanol to give the target compound **7**.

Yield 71%, mp. 264-266oC, white powder; IR (KBr, cm^{-1}): 3325, 3211 (NH_2), 3056 (CH-arom.), 2959, 2927, 2870 (CH-aliph.), 2189 (CN); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 0.64 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 1.97-2.02 (m, 1H, CH_2), 2.14-2.26 (m, 2H, CH_2), 2.46 (br.s, 1H, CH_2), 4.69 (s, 1H, pyran-H4), 6.99 (s, 2H, NH_2, D_2O exchangeable), 7.25-7.32 (m, 4H, Ar-H), 7.49 (t, 2H, ^3J = 7.8 Hz, Ar-H), 7.64 (t, 2H, ^3J = 7.8 Hz, Ar-H), 7.89 (d, 2H, ^3J = 7.8 Hz, Ar-H), 8.53 (s, 1H, pyrazole-H5); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 26.587 ((chromene-C4), 26.966 (CH_3), 28.861 (CH_3), 31.997 (chromene-C7), 50.515 (CH_2), 57.958 (CH_2), 104.172, 111.691, 112.018, 118.626, 120.440, 121.700, 123.675, 124.974, 126.728, 127.082, 128.710, 129.203, 130.097, 139.546, 141.674, 150.616, 154.682, 159.108, 163.006 (aromatic-C), 196.343 (CO); MS, m/z (%): 476 [M^+] (3), 410 [$C_{24}H_{18}N_4O3$] (58), 326 [$C_{21}H_{16}N_3O$] (85), 66 [C_5H_6] (100); Anal. For $C_{29}H_{24}N_4O_3$ (476.53): Calcd. C, 73.09; H, 5.08; N, 11.76; Found: C, 73.34; H, 5.28; N, 11.54.

6.1.6. 6-Amino-4-(3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (8)

A mixture of compound **2** (0.68 g, 0.002 mol) and 3-methyl-1*H*-pyrazol-5(4*H*)-one (0.20 g, 0.002 mol) in absolute ethanol (20 mL) containing 2-3 drops of piperidine was stirred at room temperature overnight. The formed precipitate was filtered, dried, and recrystallized from ethanol to give the target compound **8**.

Yield 75%, mp. 226-228oC, white powder; IR (KBr, cm^{-1}): 3393, 3306, 3176 (NH, NH_2), 3054 (CH-arom.), 2922, 2858 (CH-aliph.), 2186 (CN); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 1.83 (3H, s, CH_3), 5.17 (1H, s, pyran-H4), 6.89 (2H, s, NH_2, D_2O exchangeable), 7.21 (s,1H, Ar-H), 7.24-7.37 (m, 3H, Ar-H), 7.53 (t, 2H, ^3J = 7.95 Hz, Ar-H), 7.64 (t, 2H, ^3J = 7.8 Hz, Ar-H), 7.96 (d, 2H, ^3J = 7.5 Hz, ^4J = 1.2 Hz, Ar-H), 8.60 (1H, s, pyrazole-H5), 12.00 (1H, s, NH, D_2O exchangeable); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 10.251 (CH_3), 27.060 (pyran-C4), 56.927 (pyran-C3), 97.453 (CN), 104.033, 111.789, 118.689, 121.529, 121.662, 123.722, 125.010, 125.805, 127.158, 128.550, 129.216, 129.231, 130.125, 136.024, 139.572, 141.811, 150.512, 154.532, 155.337, 161.611, 171.665 (aromatic-C); MS, m/z (%): 434 [M^+] (5), 368 [$C_{22}H_{16}N_4O_2$] (100); Anal. For $C_{25}H_{18}N_6O_2$ (434.45): Calcd. C, 69.11; H, 4.18; N, 19.34; Found: C, 69.32; H, 4.35; N, 19.16.

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6.1.7. 8-(3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-6-oxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile (9)

A mixture of compound **4** (0.87 g, 0.002 mol) and 1,2-dibromoethane (0.18 mL, 0.002 mol) in pyridine (15 mL) was refluxed for 6h. The reaction mixture was poured onto ice/cold water; the formed precipitate was filtered, dried and recrystallized from ethanol to give the title compound **9**.

Yield 93%, mp. 238-240°C, white powder; IR (KBr, cm-1): 3189, 3127 (2NH), 3049 (CH-arom.), 2922, 2854 (CH-aliph.), 2213 (2CN), 1639 (C=O); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 2.07 (m, 2H, CH_2), 2.43 (m, 2H, CH_2), 6.94 (s, 1H, Ar-H), 7.24 (d, 1H, ^3J = 7.5 Hz, Ar-H), 7.32 (t, 2H, ^3J = 7.5 Hz, Ar-H), 7.42 (t, 1H, ^3J = 7.2 Hz, Ar-H), 7.58-7.67 (m, 3H, Ar-H), 7.98-8.06 (m, 2H, Ar-H), 9.05 (1H, s, pyrazole-H5), 9.09 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 31.175 (CH_2), 71.449 (CH_2), 78.486, 85.057, 105.138, 111.701, 116.926, 117.184, 119.007, 122.056, 123.729, 125.511, 127.579, 127.737, 128.670, 130.149, 130.329, 139.285, 141.722, 142.953, 146.058, 148.776, 152.938, 154.457, 156.342 (aromatic-C), 161.636 (CO); MS, m/z (%): 458 [M^+-1] (16), 457 [M^+-2] (20), 433 [M^+-C_2H_2] (40), 51 [C_4H_3] (100); Anal. For $C_{26}H_{17}N_7O_2$ (459.46): Calcd. C, 67.97; H, 3.73; N, 21.34; Found: C, 67.64; H, 3.51; N, 21.58.

6.1.8. 3-Acetyl-7-(3-(benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-3,5-dihydro-2-methyl-5-oxo-[1,2,4]triazolo[1,5-a]pyridine-6,8-dicarbonitrile (10)

A mixture of compound **4** (0.87 g, 0.002 mol) and acetic anhydride (10 mL) was refluxed for 1h. Upon cooling, the formed precipitate was filtered, dried and recrystallized from acetic acid to give the title compound **10**.

Yield 74%, mp. >300°C, yellow powder; IR (KBr, cm-1): 3051 (CH-arom.), 2925, 2846 (CH-aliph.), 2223 (2CN), 1671 (2C=O); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 1.92 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 7.04 (s, 1H, Ar-H), 7.26 (t, 1H, ^3J = 7.65 Hz, Ar-H), 7.35 (t, 1H, ^3J = 8.1 Hz, Ar-H), 7.44 (t, 1H, ^3J = 7.35 Hz, Ar-H), 7.59-7.65 (m, 4H, Ar-H), 8.02 (d, 2H, ^3J = 7.8 Hz, Ar-H), 9.09 (s, 1H, pyrazole-H5); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 21.567 (CH_3), 31.194 (CH_3), 77.169, 87.085, 105.393, 111.785, 116.187, 116.622, 117.832, 119.115, 122.038, 123.794, 125.591, 127.872, 128.696, 130.367, 139.242, 141.733, 147.311, 148.599, 151.330, 154.520, 155.657 (aromatic-C), 158.983 (CO); MS, m/z (%): 498 [M^+-1] (3), 418 [$C_{24}H_{14}N_6O_2$] (32), 56 [C_4H_8] (100); Anal. For $C_{28}H_{17}N_7O_3$ (499.48): Calcd. C, 67.33; H, 3.43; N, 19.63; Found: C, 67.52; H, 3.14; N, 19.80.

6.1.9. 7-(3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-1,2,3,5-tetrahydro-2-(substituted)-5-oxo-[1,2,4]triazolo[1,5-a]pyridine-6,8-dicarbonitrile 11a-d

A mixture of compound **4** (0.87 g, 0.002 mol) and the appropriate aldehyde derivatives namely; 3,4,5-trimethoxybenzaldehyde, 4-chlorobenzaldehyde, 5-methylfuran-2-carbaldehyde and/or thiophene-2-carbaldehyde (0.002 mol) in acetic acid (20 mL) was refluxed for 6-8h. After cooling, the formed precipitate was filtered, dried and recrystallized from acetic acid to give the title compounds **11a-d** respectively.

6.1.9.1. 7-(3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-1,2,3,5-tetrahydro-2-(3,4,5-trimethoxyphenyl)-5-oxo-[1,2,4]triazolo[1,5-a]pyridine-6,8-dicarbonitrile (11a)

Yield 83%, mp. 297-299°C, white powder; IR (KBr, cm-1): 3299, 3214 (2NH), 3067 (CH-arom.), 2942, 2832 (CH-aliph.), 2221 (2CN), 1665 (C=O); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 3.79 (s, 3H, OCH3), 3.89 (s, 6H, 2(OCH3)), 7.27-7.48 (m, 6H, Ar-H), 7.63 (t, 2H, J = 7.95 Hz, Ar-H), 7.71 (d, 2H, J = 7.80 Hz, Ar-H), 8.03 (d, 2H, J = 7.80 Hz, Ar-H), 8.56 (br, 2H, 2NH, D_2O exchangeable), 8.90 (s, 1H, triazole-H3), 9.13 (s, 1H, pyrazole-H5); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 56.65, 60.82, 90.26, 107.80, 115.75, 115.77, 119.21, 122.18, 125.63, 125.70, 125.74, 125.76, 125.79, 126.23, 126.38, 126.73, 127.10, 127.39,127.40, 127.73, 128.77, 130.43, 140.88, 146.02, 147.29, 153.53, 154.61, 156.86, 158.59, 159.06, 159.42 (aromatic-C); MS, m/z (%): 611 [M^+] (74), 610 [M^+-1] (29), 236 [$C_{11}H_{14}N_3O_3$] (100); Anal. For $C_{34}H_{25}N_7O_5$ (611.62): Calcd. C, 66.77; H, 4.12; N, 16.03; Found: C, 66.41; H, 4.33; N, 16.35.

6.1.9.2. 7-(3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-2-(4-chlorophenyl)-1,2,3,5-tetrahydro-5-oxo-[1,2,4]triazolo[1,5-a]pyridine-6,8-dicarbonitrile (11b)

Yield 61%, mp. 221-223°C, white powder; IR (KBr, cm-1): 3299, 3192 (2NH), 3055 (CH-arom.), 2218 (2CN), 1676 (C=O); ^{1}H NMR (300 MHz; DMSO-d_6) δ_H 7.28-7.32 (m, 2H, Ar-H), 7.38 (t, 1H, J = 7.2

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Hz, Ar-H), 7.45 (t, 1H, *J* = 7.35 Hz, Ar-H), 7.45 (t, 2H, *J* = 7.95 Hz, Ar-H), 7.68 (d, 4H, *J* = 8.4 Hz, Ar-H), 7.99 (d, 2H, *J* = 7.8 Hz, Ar-H), 8.09 (d, 2H, *J* = 8.7 Hz, Ar-H), 8.62 (br., 2H, 2NH, D_2O exchangeable), 9.07 (s, 1H, triazole-CH), 9.12 (s, 1H, pyrazole-H5) ; ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 76.499, 89.110, 105.763, 111.916, 111.946, 115.736, 116.647, 119.239, 122.067, 123.909, 123.939, 125.715, 128.070, 128.093, 128.762, 129.567, 130.421, 131.154, 132.048, 138.544, 139.117, 148.353, 154.634, 156.809, 161.978, 170.867, 171.920 (aromatic-C); MS, m/z (%): 555 [M+] (3), 556 [M^{+1}] (2), 418 [$C_{24}H_{14}N_6O_2$] (58), 137 [C_7H_4ClN] (100); Anal. For $C_{31}H_{18}ClN_7O_2$ (555.97): Calcd. C, 66.97; H, 3.26; N, 17.64; Found: C, 67.19; H, 3.42; N, 17.39.

6.1.9..3. 7-(3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-1,2,3,5-tetrahydro-2-(5- methylfuran-2-yl)-5-oxo-[1,2,4]triazolo[1,5-a]pyridine-6,8-dicarbonitrile (11c)

Yield 85%, mp. 217-219°C, grey powder; IR (KBr, cm-1): 3196, 3125 (2NH), 3062 (CH-arom.), 2929, 2850 (CH-aliph.), 2217 (2CN), 1664 (C=O); ^1H NMR (300 MHz; DMSO-d_6) δ_H 2.46 (s, 3H, CH_3), 6.51 (d, 1H, *J* = 6.7 Hz, furan-H), 6.95 (s, 1H, furan-H), 7.17 (s, 1H, Ar-H), 7.24-7.47 (m, 4H, Ar-H), 7.62-7.69 (m, 2H, Ar-H), 7.99-8.01 (m, 2H, Ar-H), 8.44, 8.58 (2br., 2H, 2NH, D_2O exchangeable), 8.68 (s, 1H, pyrazole-H5), 9.11 (s, 1H, triazole-CH); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 14.276 (CH_3), 40.837, 69.025, 76.386, 105.012, 111.897, 115.786, 119.199, 122.049, 125.689, 128.717, 130.398, 139.103, 141.601, 146.325, 154.605, 157.165, 159.197, 165.013, 177.823**;** MS, m/z (%): 525 [M+] (21), 526 [M++1] (10), 82 [C_5H_6O] (100); Anal. For $C_{30}H_{19}N_7O_3$ (525.52): Calcd. C, 68.57; H, 3.64; N, 18.66; Found: C, 68.73; H, 3.49; N, 18.40.

6.1.9.4. 7-(3-(Benzofuran-2-yl)-1-phenyl-1H-pyrazol-4-yl)-1,2,3,5-tetrahydro-5-oxo-2-(thiophen-2-yl)-[1,2,4]triazolo[1,5-a]pyridine-6,8-dicarbonitrile (11d)

Yield 92%, mp. 194-196°C, grey powder; IR (KBr, cm-1): 3214, 3136 (2NH), 3051 (CH-arom.), 2927, 2845 (CH-aliph.), 2219 (2CN), 1668 (C=O); ^1H NMR (300 MHz; DMSO-d_6) δ_H 7.10-8.15 (14H, m, Ar-H, triazole-H3), 8.50, 9.01 (2H, 2s, 2NH, D2O exchangeable), 9.12 (1H, s, pyrazole-H5); ^{13}C NMR (75 MHz; DMSO-d_6) δ_C 75.913, 88.118, 105.324, 111.748, 119.105, 121.996, 123.847, 125.628, 127.974, 128.563, 130.344, 139.048, 141.492, 148.439, 151.319, 156.825, 159.667, 171.609**;** MS, m/z (%): 527 [M^+] (42), 528 [M^{+1}] (41), 443 [M^+- C_4H_4S] (45), 77 [C_6H_5] (100); Anal. For $C_{29}H_{17}N_7O_2S$ (527.56): Calcd. C, 66.02; H, 3.25; N, 18.59; S, 6.08; Found: C, 66.28; H, 3.41; N, 18.72; S, 6.22.

5.2. Biological Evaluation

5.2.1. In Vitro Antimicrobial Activity

The antibacterial screening bioassay was made by the agar well diffusion method using Mueller-Hinton agar (Lab M Limited, Bury, Lancashire, UK), then the plates were transferred to refrigerator for 1 h at 4 °C [49–52]. More detailed descriptions are available in the file of Supporting Information.

5.2.2. Human Red Blood Cell Stabilization Method

The human red blood cell (HRBC) membrane stabilization method was used to study the *in vitro* anti-inflammatory activity of the new samples [69]. More detailed descriptions are available in the file of Supporting Information.

5.2.3. DPPH Radical Scavenging Assay

The DPPH (1–diphenyl–2–picrylhydrazyl) scavenging activity of the sample was determined quantitatively according to the reported method [70,71]. More detailed descriptions are available in the file of Supporting Information.

5.2.4. E. coli DNA Gyrase B Suppression Effect

The *in vitro* enzyme inhibition assessment was performed against *E. coli* DNA gyrase according to the optimized protocol by the manufacturer [62–64]. More detailed descriptions are available in the file of Supporting Information.

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5.2.5. In Vitro Cytotoxicity Assay

The cytotoxic effect of test samples using WI38 cells was evaluated by MTT assay [64]. Commercially available kit for in vitro toxicology MTT based assay, Sigma was used. More detailed descriptions are available in the file of Supporting Information.

5.2.6. Molecular Docking

Molecular Operating Environment (MOE, 2019.0102)[1] was used for the docking study. By using the steepest descent technique with the MMFF94x force field until the RMSD gradient of 0.1 kcal.mol^{-1}Å^{-1} was reached, energy-minimized structures were created. The crystal structure of DNA gyrase enzyme complexed with novobiocin was obtained from the protein data bank[1] with PDB ID: **4URO**. In the same way, the x-ray crystallographic structure of topoisomerase IV with the co-crystallized ligand; novobiocin; was downloaded (PDB ID: **1S14**). Despite docking software's capacity to identify potential binding modes between a ligand and its target, it is still an unreliable method that requires constant verification[1] [66–68].

CRediT authorship contribution statement.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, Somaia Abd El-Karima; Data curation, Somaia Abd El-Karima, Manal Anwara, Yasmin Syama, Hassan Awad, Asmaa El-Dein and Mohamed El-Ashrey; Formal analysis, Yasmin Syama, Asmaa El-Dein, Mohamed El-Ashrey and Sameh Abdelwahed; Investigation, Somaia Abd El-Karima and Yasmin Syama; Methodology, Somaia Abd El-Karima, Manal Anwara, Mohamed El-Ashrey and Sameh Abdelwahed; Project administration, Somaia Abd El-Karima, Manal Anwara and Sameh Abdelwahed; Resources, Somaia Abd El-Karima, Yasmin Syama, Asmaa El-Dein and Sameh Abdelwahed; Software, Mohamed El-Ashrey; Supervision, Somaia Abd El-Karima, Manal Anwara and Sameh Abdelwahed; Validation, Somaia Abd El-Karima, Hassan Awad, Mohamed El-Ashrey and Sameh Abdelwahed; Visualization, Somaia Abd El-Karima, Hassan Awad and Mohamed El-Ashrey; Writing – original draft, Somaia Abd El-Karima, Manal Anwara, Yasmin Syama and Sameh Abdelwahed; Writing – review & editing, Somaia Abd El-Karima, Manal Anwara, Yasmin Syama, Hassan Awad, Asmaa El-Dein, Mohamed El-Ashrey and Sameh Abdelwahed.

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