Novel heterocyclic hybrids of pyrazole targeting dihydrofolate reductase: design, biological evaluation and in silico studies

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ABSTRACT
A novel series of pyrazole analogues including hydrazones, pyrazolo[4,3-c]-pyridazines, pyrazolo[3,4-e][1,2,4]triazine and pyrazolo[3,4-d][1,2,3]triazoles was designed, synthesised and screened for their in vitro antimicrobial and DHFR inhibition activity. Compounds bearing benzensulphonamide moiety incorporated with 3-methyl-5-oxo-1H-pyrazol-4(5H)-ylidene hydrazine 3a or 6-amino-7-cyano-3-methyl-5H-pyrazolo[4,3-c]pyridazine 6a revealed excellent and broad spectrum antimicrobial activity comparable to ciprofloxacin and amphotericin B as positive antibiotic and antifungal controls, respectively. Furthermore, these derivatives proved to be the most active DHFR inhibitors with IC$_{50}$ values 0.11 ± 1.05 and 0.09 ± 0.91 µM, in comparison with methotrexate (IC$_{50}$ = 0.14 ± 1.25 µM). The in silico studies were done to calculate the drug-likeness and toxicity risk parameters of the newly synthesised derivatives. Additionally, the high potency of the pyrazole derivatives bearing sulphonamide against DHFR was confirmed with molecular docking and might be used as an optimum lead for further modification.

Introduction
The increased microbial resistance has led to the demand for new bactericidal and fungicidal agents. The reasons for resistance are the inaccurate diagnosis as well as misuse and widespread use of antimicrobial agents. So, a worldwide effort to search for new generation drugs was stimulated to get new potent, resistance-free, and safer antimicrobial agents.

Recently, dihydrofolate reductase (DHFR) has been considered to be a universal and attractive enzyme which is present in all organisms. Its essential function is to catalyse the reduction of dihydrofolate to tetrahydrofolate within the thymidylate synthesis cycle. As a result, inhibition of DHFR causes "thymineless death". Inhibitors of DHFR explored a crucial role in medicine like methotrexate that is a non-selective inhibitor and a confirmed agent used in oncology for the treatment of rheumatoid arthritis and several cancers. Thus, there is a vast number of interesting target profiles and literatures achievable with DHFR and its inhibitors.

Pyrazoles are a class of interesting heterocyclic compounds characterised by the presence of two nitrogen atoms adjacent to three carbon atoms in five-membered aromatic ring structure. Pyrazole derivatives play an imperative role in wide spectrum of biological activities including antibacterial, antifungal, anti-inflammatory, analgesic, oestrogen receptor binding, neuropeptidergic, antineoplastic and many others. Furthermore, several reports exhibited that pyrazoles I–IV and pyrazolo[3,4-d]pyrimidine V (Figure 1) had high bactericidal and fungicidal activity against the reference strains. Additionally, the pyrazole-containing cores (VI–VII) were found to be active as antimicrobial and anti-malarial through inhibition of DHFR (Figure 2). Regarding to their broad spectrum of biological activities, pyrazole ring has been considered as a favourable unit for addition in the field of drug discovery and therefore in the pharmaceutical industry.

In addition, several pharmacologically active structural units, for example, pyridazines, 1,2,4-triazines and 1,2,3-triazoles are being explored to identify novel lead antimicrobial molecules (Figure 3). The pyridazine derivative IX revealed promising in vitro antimicrobial activities against Gram +ve and Gram -ve bacteria in comparison with tetracycline. Nagawade et al. reported that the pyridazine-3-carboxylic acid analogue X showed gratifying in vitro antibacterial results approaching that of corresponding reference, ciprofloxacin. Compound containing 1,2,4-triazine nucleus XI has been reported to possess better antimicrobial activity with less toxicity than ciprofloxacin. The derivative bearing 1,2,3-triazole scaffold XIIa displayed overall encouraging efficiency against all screened microbial strains except Aspergillus niger, while the other derivative XIIb exhibited better antimicrobial potency against all strains except Bacillus subtilis and Aspergillus niger.

Motivated by the aforementioned findings, and upon continuation of our research programme in the field of discovery of pyrazole bearing antimicrobial agents, some novel pyrazole prototypes were designed and synthesised after exploring...
molecular fusion with pyridazine, triazine and triazole moieties. The synthesised compounds comprising pyrazole motif were evaluated in vitro for their antimicrobial activities against human pathogenic microbes and their inhibitory activities against DHFR enzyme. Finally, molecular docking and in silico studies were used to explain the obtained biological data.

Experimental

Chemistry

All melting points were determined on a Gallenkamp apparatus and are uncorrected. The IR spectra were measured on a Pye-UnicamSP300 instrument in potassium bromide discs. The $^1$H-NMR and $^{13}$C-NMR spectra were recorded on Varian Mercury VX (300 MHz) spectrometer (with operating frequencies 300.07 MHz for $^1$H using TMS as an internal standard and 75.45 MHz for $^{13}$C). Chemical shifts ($\delta$) are reported in parts per million (ppm), and coupling constants ($J$) are reported in Hertz (Hz). NMR spectra were recorded at temperature (30–45 °C) and were referenced to the residual signals of DMSO- $d_6$. Elemental analyses were carried out by the Micro analytical Centre of Cairo University, Giza, Egypt. The antimicrobial activities were carried out in the Medical Mycology Laboratory of the Regional Centre for Mycology and Biotechnology of Al-Azhar University, Cairo, Egypt.

General procedure for preparation of hydrazones 3a and 3b

To a solution of 1 (1 g, 10 mmol) and sodium acetate (1.64 g, 20 mmol) in absolute ethanol (20 ml), the appropriate diazonium salt of aromatic amines 2a and 2b (10 mmol) [prepared by diazotising a solution of each of sulphanimide and 1-naphthyl amine, respectively (10 mmol) in hydrochloric acid (1.5 ml) with a solution of sodium nitrite (0.69 g, 10 mmol) in 5 ml water in ice bath], was added portion wise with stirring at 0–5 °C. The reaction mixture was stirred for further 1 h and diluted with water. The obtained precipitate was collected by filtration, washed with H$_2$O and recrystallised from ethanol to afford the corresponding hydrazine derivatives 3a and 3b, respectively.
Figure 3. Reported antimicrobial leads containing pyridazine, 1,2,4-triazine and 1,2,3-triazole moieties.

4-(2-(3-Methyl-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene)hydrazineyl)benzenesulphonamide (3a)

Yield: 79%; yellow crystals; m.p. 201–203 °C; IR (KBr) ν cm⁻¹ 3472, 3346 (NH₃), 3235, 3120 (2NH), 3057 (CH-arom.), 2925 (CH-aliph.), 1733 (CO), 1367, 1145 (SO₂); ¹H NMR (DMSO-d₆) δ = 2.61 (s, 3H, CH₃), 6.44 (s, 2H, NH₂, exchangeable with D₂O), 7.19 – 7.2 22 (d, J = 7.2 Hz, 2H, Ar-H), 7.60 – 7.62 (d, J = 7.2 Hz, 2H, Ar-H), 9.15 (s, 1H, NH, exchangeable with D₂O), 12.37 (s, 1H, NH-hydrazone); ¹³C NMR (DMSO-d₆): 12.9, 117.8, 127.4, 130.3, 130.6, 146.1, 149.1, 164.5. Anal. Calcd. for C₁₀H₁₁N₅O₃S (281.29): C, 42.70; H, 3.94; N, 24.90; S, 11.40%. Found: C, 42.51; H, 3.72; N, 24.73; S, 11.75%.

5-Methyl-4-(2-(naphthalene-1-yl)hydrazineylidene)-2,4-dihydro-3H-pyrazol-3-one (3b)

Yield 80%; pale yellow crystals; m.p. 189–191 °C; IR (KBr) ν cm⁻¹ 3368, 3242 (2NH), 3071 (CH-arom.), 2948 (CH-aliph.), 1730 (CO); ¹H NMR (DMSO-d₆) δ = 2.48 (s, 3H, CH₃), 7.34 – 7.66 (m, 6H, naphthyl-H), 8.18 – 8.21 (d, J = 8.1 Hz, 1H, naphthyl-H), 9.16 (s, 1H, NH, exchangeable with D₂O), 12.66 (s, 1H, NH-hydrazine, exchangeable with D₂O); ¹³C NMR (DMSO-d₆): 14.1, 106.3, 120.2, 122.1, 122.3, 124.2, 125.5, 127.1, 129.4, 131.0, 143.1, 147.2, 182.2. Anal. Calcd. for C₁₄H₁₂N₄O (252.27): C, 66.65; H, 4.79; N, 22.21%. Found: C, 66.88; H, 4.57; N, 22.43%.

General procedure for preparation of compounds 6a–d

A mixture of 3a (2.81 g, 10 mmol) with each of either malononitrile (4a) (0.66 g, 10 mmol) or ethyl cyanoacetate (4b) (1.13 g, 10 mmol) was used in the presence of ammonium acetate (1.54 g, 20 mmol) at 150 °C for 30 min, then left to stand at room temperature and triturated with absolute ethanol. The solid products so formed were collected by filtration and recrystallised from ethanol/DMF to give 6a and 6b, respectively.

Similarly, a mixture of 3b (2.52 g, 10 mmol) with each of either malononitrile (4a) (0.66 g, 10 mmol) or ethyl cyanoacetate (4b) (1.13 g, 10 mmol) was used in the presence of ammonium acetate (1.54 g, 20 mmol) at 150 °C for 30 min, then left to stand at room temperature and triturated with absolute ethanol. The solid products so formed were collected by filtration and recrystallised from ethanol/DMF to give 6c and 6d, respectively.

4-(6-Amino-7-cyano-3-methyl-5H-pyrazolo[4,3-c]pyridazin-5-yl)benzenesulphonamide (6a)

Yield: 68%; green crystals; m.p. 298–300 °C; IR (KBr) ν cm⁻¹ 3441, 3357, 3232, 3125 (2NH), 3054 (CH-arom.), 2933 (CH-aliph.), 2205 (C=O), 1336, 1174 (SO₂); ¹H NMR (DMSO-d₆) δ = 2.77 (s, 3H, CH₃), 6.87 (s, 2H, NH₂, exchangeable with D₂O), 7.28 – 7.30 (J = 7.2 Hz, d, 2H, Ar-H), 8.08 – 8.10 (J = 7.2 Hz, d, 2H, Ar-H), 8.69 (s, 2H, NH₂, exchangeable with D₂O); ¹³C NMR (DMSO-d₆): 11.1, 82.1, 115.0, 116.1, 129.2, 131.9, 137.8, 140.6, 147.8, 155.3, 162.6. Anal. Calcd. for C₁₃H₁₁N₅O₃S (329.34): C, 47.41; H, 3.37; N, 29.77; S, 9.74%. Found: C, 47.62; H, 3.58; N, 29.54; S, 9.95%.

4-(7-Cyano-3-methyl-6-oxo-1,6-dihydro-5H-pyrazolo[4,3-c]pyridazin-5-yl)benzenesulphonamide (6b)

Yield: 66%; brown crystals; m.p. 294–296 °C; IR (KBr) ν cm⁻¹ 3437, 3329 (NH₂), 3241 (NH), 3051 (CH-arom.), 2943 (CH-aliph.), 2207 (C=O), 1728 (CO), 1341, 1162 (SO₂); ¹H NMR (DMSO-d₆) δ = 2.25 (s, 3H, CH₃), 6.72 (s, 2H, NH₂, exchangeable with D₂O), 7.77 – 7.79 (d, J = 7.2 Hz, 2H, Ar-H), 8.10 – 8.13 (d, J = 7.2 Hz, 2H, Ar-H), 9.60 (s, 1H, NH, exchangeable with D₂O); ¹³C NMR (DMSO-d₆): 10.6, 78.3, 115.6, 120.2, 130.5, 134.9, 138.7, 142.3, 148.2, 160.1, 169.7. Anal. Calcd. for C₁₃H₁₀N₄O₃S (330.32): C, 47.27; H, 3.05; N, 25.44; S, 9.71%. Found: C, 47.48; H, 3.28; N, 25.66; S, 9.92%.

6-Amino-3-methyl-5-(naphthalene-1-yl)-5H-pyrazolo[4,3-c]pyridazine-7-carbonitride (6c)

Yield 65%; brown crystals; m.p. 288–290 °C; IR (KBr) ν cm⁻¹ 3328, 3232 (NH₂), 3086 (CH-arom.), 2944 (CH-aliph.), 2215 (C=O); ¹H NMR (DMSO-d₆) δ = 2.60 (s, 3H, CH₃) 7.33 – 7.62 (m, 9H, Ar-H + NH₂,
exchangeable with D$_2$O); $^{13}$C NMR (DMSO-d$_6$): 101.1, 99.7, 107.5, 115.0, 118.3, 120.8, 121.0, 125.0, 127.7, 128.5, 129.5, 131.2, 139.7, 141.2, 149.9, 155.5, 162.6. Anal. Calcd. for C$_{17}$H$_{14}$N$_6$O$_2$S$_2$ (398.46): C, 67.56; H, 3.89; N, 23.47%. Found: C, 67.78; H, 4.23; N, 27.75%.

3-Methyl-5-(naphthalen-1-yl)-6-oxo-5,6-dihydro-3H-pyrazolo[4,3-c]pyridazine-7-carbonitrile (6d)

Yield 61%; brown crystals; m.p. > 300°C; IR (KBr) ν cm$^{-1}$ 3381, 3257 (NH$_2$), 3131 (SO$_2$); $^1$H NMR (DMSO-d$_6$) δ = 2.89 (s, 3H, CH$_3$), 6.31 (s, 2H, NH$_2$), exchangeable with D$_2$O); $^{13}$C NMR (DMSO-d$_6$): 21.6, 124.2, 127.11, 127.14, 129.4, 132.0, 134.5, 136.2, 137.9, 143.5, 151.4, 157.6, 176.1. Anal. Calcd. for C$_{17}$H$_{14}$N$_6$O$_2$S$_2$ (398.46): C, 51.24; H, 3.54; N, 21.09; S, 16.09%. Found: C, 51.55; H, 3.75; N, 21.32; S, 16.30%.

General procedure for preparation of compounds 9a and 9b

Dissolve either (2.81 g, 10 mmol) of 3a or (2.52 g, 10 mmol) of 3b in dimethylformamide (30 ml), and then add 0.5 ml of triethylamine. Each of the solutions was treated with hydroxylamine hydrochloride (0.69 g, 10 mmol). The reaction mixtures were heated under reflux for 24 h then left to cool, and poured into crushed ice and acidified with 10% HCl. The solid product obtained was collected by filtration and recrystallised from dioxan/ethanol to give 9a and 9b, respectively.

4-(6-Methylpyrazolo[3,4-d][1,2,3]triazol-2(4H)-yl)benzenesulphonamide (9a)

Yield: 63%; pale yellow crystals; m.p. > 300°C; IR (KBr) ν cm$^{-1}$ 3381, 3257 (NH$_2$), 3136 (NH), 3077 (CH-arom.), 2925 (CH-aliph.), 1385, 1311 (SO$_2$); $^1$H NMR (DMSO-d$_6$) δ = 2.89 (s, 3H, CH$_3$), 6.31 (s, 2H, NH$_2$), exchangeable with D$_2$O); $^{13}$C NMR (DMSO-d$_6$): 7.54 – 7.57 (d, J = 7.2 Hz, 2H, Ar-H), 7.64 – 7.66 (d, J = 7.2 Hz, 2H, Ar-H), 12.62 (s, 1H, NH, exchangeable with D$_2$O); $^{13}$C NMR (DMSO-d$_6$): 10.2, 127.8, 129.4, 134.2, 141.9, 143.9, 146.1. Anal. Calcd. for C$_{14}$H$_{12}$N$_2$O$_2$S (278.29): C, 43.16; H, 3.62; N, 30.20; S, 11.52%. Found: C, 43.37; H, 3.84; N, 30.41; S, 11.75%.

6-Methyl-2-(naphthalen-1-yl)-2,4-dihydropyrazolo[3,4-d][1,2,3]triazole (9b)

Yield: 67%; pale yellow crystals; m.p. > 300°C; IR (KBr) ν cm$^{-1}$ 3292 (NH), 3086 (CH-arom.), 2944 (CH-aliph.); $^1$H NMR (DMSO-d$_6$) δ = 2.72 (s, 3H, CH$_3$) 7.40 – 8.20 (m, 7H, Ar-H), 12.76 (s, 1H, NH, exchangeable with D$_2$O); $^{13}$C NMR (DMSO-d$_6$): 10.3, 123.2, 123.5, 125.5, 125.9, 127.3, 129.8, 130.6, 132.1, 134.5, 139.1, 146.1. Anal. Calcd. for C$_{17}$H$_{15}$N$_5$ (249.27): C, 67.46; H, 4.45; N, 28.10%. Found: C, 67.46; H, 4.45; N, 28.10%.
Biological activity

Antimicrobial activity sensitivity assay

The target compounds 3–9 were screened in vitro opposite to various types of bacteria, *Streptococcus pneumoniae* and *Bacillus subtilis* as examples of Gram-positive bacteria, and *Pseudomonas aeruginosa* and *Escherichia coli* as examples of Gram-negative bacteria and for their Antifungal activities against *Aspergillus fumigates* and *Candida albicans*, respectively. Solutions of concentrations (1 μg/mL) of the target compounds were used. The agar media were inoculated with different microorganism’s culture tested after 24 h of inoculation at 37°C for bacteria and for antifungal tested after 72 h of inoculation at 28°C. Ciprofloxacin and amphotericin B were used as standard antibacterial and antifungal drugs, respectively. The diameter of inhibition zone (mm) was measured for the biologically activity using the diffusion technique. The active compounds 3a, 3b, 6a, 6b, 8a and 9a were further investigated to determine their antimicrobial activity expressed in terms of minimum inhibitory concentration (MIC) using the modified agar well diffusion method that mentioned above. The different concentrations (triplicate) of each compound were tested and compared with standard drugs.

Dihydrofolate reductase (DHFR) inhibition

The newly synthesised active targets 3a, 3b, 6a, 6b, 8a and 9a were assessed for their in vitro inhibition against dihydrofolate reductase (DHFR) in confirmatory diagnostic unit, Vacsera, Egypt. Methotrexate was used as a reference drug following the previously mentioned method. The obtained results are depicted as IC₅₀ values of enzyme inhibition in Table 2. The assay mixture contained 50 μM Tris–HCl buffer (pH 7.4), 50 μM NADPH, 10 μM DMSO or the same volume of DMSO solution containing the test compounds to a final concentration of 10⁻¹¹ to 10⁻⁸ M, and 10 μl of DHFR, in a final volume of 1.0 ml. After addition of the enzyme, the mixture was incubated at room temperature for 2.0 min, and the reaction was initiated by adding 5 μl of dihydrofolic acid, the change in absorbance (ΔOD/min) was measured by the spectrophotometer at 340 nm and 22°C, kinetic programme (reading every 15 s for 2.5 min). Results are reported as % inhibition of enzymatic activity calculated using the following formula:

\[
\text{Fractional activity of enzyme} = \left( \frac{\text{Sample } \Delta \text{OD/min} - \text{ blank } \Delta \text{OD/min}}{\text{d}} \times \frac{Vx}{3x} \times \frac{mgP}{mL} \right) \times \frac{P}{mgP/mL} 
\]

where
- ΔOD/min: the spectrophotometer readings
- 12.3: extinction coefficient for the DHFR reaction at 340 nm.
- V: Enzyme volume in mL (the volume of enzyme used in the assay)
- d: The dilution factor of the enzyme sample.
- mgP/mL: enzyme concentration of the original sample before dilution.

In silico calculations of molecular properties

Molecular descriptors express the pharmacokinetic, pharmacodynamic and physicochemical effects of all newly synthesised targets 3, 6, 8 and 9. The lipophilicity (milogP) and topological polar surface area (tPSA) were measured using the online Molinspiration software while the drug score, drug-likeness and aqueous solubility were evaluated via the OSIRIS property explorer software. Furthermore, good bioavailability is more preferable for targets having TPSA of ≤20 Å² and ≤10 rotatable bonds.

Molecular modelling study

The molecular docking a powerful tool to understanding and rationalise the obtained biological results. The interactions of the

Figure 5. 2D and 3D Views (A, B) of the original ligand, methotrexate re-docked in the active site of DHFR (PDB ID: 1DLS) using MOE software. 3D representation (C) of the superimposition of the docking pose (yellow) and the co-crystallised (red) of methotrexate with an RMSD of 0.88 Å.
newly synthesised compounds 3a and 6a having the highest DHFR inhibitory activity were investigated with the active site of the target kinase to study their mode of binding and orientations. All the molecular modelling studies were carried out using MOE, 10.2008 software. All minimisations were performed with MOE until an RMSD gradient of 0.05 kcal mol\(^{-1}\) Å\(^{-1}\) with MMFF94x force field and the partial charges were automatically calculated. The co-crystallised structure of the dihydrofolate reductase enzyme was downloaded from Protein Data Bank website (PDB ID: 1DLS). The enzyme structure was prepared for molecular docking using Protonate 3D protocol in MOE with the default options. The co-crystallised inhibitor was used to define the active site for molecular docking. Triangle Matcher placement method and London dG scoring function were used in the docking protocol. Docking setup was first validated by self-docking of the co-crystallised inhibitor in the enzyme active site giving a docking pose with an energy score (S) = \(-11.68\) kcal mol\(^{-1}\) and an RMSD of 0.88 Å from the co-crystallised ligand pose (Figure 5). Then, the validated molecular docking setup was used to investigate the ligand–target interactions of the newly synthesised compounds 3a and 6a in the DHFR active site to predict their binding pattern and to investigate their ability to satisfy the required structural features for binding interactions (Figures 6 and 7).

**Results and discussion**

**Chemistry**

The synthetic strategies approved for the synthesis of the intermediate and target compounds are outlined in Schemes 1 and 2. Thus, 3-methylpyrazol-5(4H)-one 1 was coupled smoothly with diazonium salts 2a and 2b in the presence of ethanol and sodium acetate at 0\(-5\) °C, to afford the respective hydrazines 3a and 3b (Scheme 1). The latter products were established on the basis of their analytical and spectral data. For example, the infrared of 6a indicated the presence of the two NH2 absorption band at \(3441, 3357, 3232, 3125\), and 2205 cm\(^{-1}\). While, its \(^1\)H NMR revealed singlet signal at \(2.77\) ppm which was assigned for CH3 group, two signals at \(6.87\) and 8.69 due to two NH protons and the absence of any signals may be attributed to NH.
Scheme 1. Synthesis of pyrazolopyridazine derivatives.

Scheme 2. Synthesis of pyrazolotriazine and pyrazolotriazole derivatives.
protons, beside aromatic protons in the molecule. The $^{13}$C-NMR of 6a showed signals at 11.1 and 115.0 for the carbons of the CH$_3$ and C=N groups, in addition to the sp$_{2}$ carbon atoms (Scheme 1 and Experimental part). The formation of 6a and 6c from 3a and 3b is assumed to proceed through the intermediate of non-isolable adducts 5a and 5c, which cyclised into the imino structure i via loss of water molecule, which tautomiser into the end amino products 6a and 6c (Scheme 1).

Similarly, 3a and 3b reacted with ethyl cyanoacetate (4b) in the same above reaction conditions to afford 6-oxyopyrazol[4,3-c]pyridazine derivatives 6b and 6d based on the elemental analysis and spectral data. The IR spectra of 6b and 6d indicate the presence of C=N absorption bands at 2207, 2217 cm$^{-1}$, respectively and C=O at 1728, 1732 cm$^{-1}$, respectively. $^1$H NMR spectra of 6b and 6d showed the presence of only one signal attributable to NH at 9.60 and 9.42 ppm, respectively. Also, its $^{13}$C NMR spectra revealed the signals of amidic C=O at 169.7 and 168.7 ppm, respectively.

The structure 6b and 6d was assumed to be formed through the non-isolable adducts 5b and 5d, which underwent cyclisation into structure ii via loss of water molecule then loss of ethanol molecule to give the end reaction products the oxo derivatives 6b and 6d (Scheme 1).

The starting material 3a and 3b used in this study was proved to be a versatile for synthesis of some novel pyrazolo[3,4-e][1,2,4]triazines and pyrazolo[3,4-d]-[1,2,3]triazoles.

Thus, 3a and 3b were reacted with phenylisothiocyanate in refluxing ethanol containing a catalytic amount of triethylamine to afford the non-isolable adducts 5b and 5d, which underwent cyclisation into structure ii via loss of water molecule then loss of ethanol molecule to give the end reaction products the oxo derivatives 6b and 6d (Scheme 1).

The infra-red spectra of 8a and 8b indicated the absence of the 2NH and carbonyl absorption bands and revealed the characteristic absorption band for the thiocarbonyl group. For example, the NH proton, in addition to the presence of aromatic protons (cf. Scheme 2 and Experimental part). The carbonyl group of pyrazole moiety was assume to be formed through the non-isolable adducts 5b and 6b, which underwent cyclisation into structure ii via loss of water molecule, then loss of ethanol molecule to give the end reaction products 8a and 8b.

Further illustration of the hydrazone structure 3 came from the reaction with hydroxyl amine hydrochloride in the presence of dimethyl formamide (DMF)/triethylamine solution under reflux to give the final product pyrazolotriazole derivative 9. The structures of 9a and 9b were in consistence with their respective analytical and spectral studies (Scheme 2).

The IR spectrum of 9a, taken as a typical example, was characterised by the disappearance of absorption band due to the CO group. Its $^1$H-NMR showed two singlet at $\delta$ 2.89, 6.31 ppm which were assigned for CH$_3$ and NH$_2$ protons, respectively and a singlet at $\delta$ 12.62 ppm attributable to the pyrazole-NH proton, in addition to the presence of aromatic protons (cf. Scheme 2 and Experimental Section). The carbonyl group of pyrazole moiety was also absent in $^{13}$C-NMR spectra.

### Biological activity

#### Antimicrobial sensitivity assay

The antimicrobial activity of all synthesised compounds 3, 6, 8 and 9 was evaluated by agar well diffusion method using ciprofloxacin and amphotericin B as standard references for antibacterial and antifungal activity, respectively. The screening was done for the in vitro antibacterial activity against Gram-positive Streptococcus pneumoniae and Bacillus subtilis, and Gram-negative Pseudomonas aeruginosa and Escherichia coli, and for the in vitro antifungal activity against Aspergillus fumigates and Candida albicans. The measure zones of inhibition are presented in Figure 4 in mm. By investigation of the given data, it was observed that compounds 3a, 3b, 6a, 6b, 8a and 9a displayed excellent inhibition zone diameter ranging from 11.6 to 28.7 mm against all tested strains in comparison with the reference drugs. On the other hand, the remaining derivatives showed weak or no antimicrobial activity.

Minimum inhibitory concentration (MIC) of the most active compound 3a, 3b, 6a, 6b, 8a and 9a was measured in vitro using twofold serial dilution technique. The results of MIC were recorded in Table 1. It was noticed that compound 6a among other derivatives revealed excellent and the highest MIC value all over the tested strains. Moreover, the derivatives 3a and 6b were equipotent with the reference, amphotericin B against A. fumigates (MIC = 1.95 $\mu$g/mL) and exhibited two folds decrease in the potency than the standard, ciprofloxacin against S. pneumonia (MIC = 1.95 and 0.98 $\mu$g/mL, respectively). Additionally, 6b was equipotent in the antifungal activity with reference against A. fumigates. All the remaining derivatives illustrated from moderate-to-weak antimicrobial activity.

### Table 1. Minimal inhibitory concentrations (MICs) of the synthesised compounds against the tested pathogenic bacteria and fungi.

| Compound No. | Gram-positive Bacteria | Gram-negative Bacteria | Fungi |
|--------------|------------------------|------------------------|-------|
|               | S. pneumonia RCMB 010010 | B. subtilis RCMB 010067 | P. aeruginosa RCMB 010043 | E. coli RCMB 010052 | A. fumigatus RCMB 002568 | C. albicans RCMB 005036 |
| 3a           | 1.95 ± 0.3             | 0.98 ± 0.1             | 7.81 ± 0.3          | 0.98 ± 0.6          | 1.95 ± 0.1           | 3.9 ± 0.3           |
| 3b           | 7.81 ± 0.1             | 15.6 ± 0.5             | 12.62 ± 0.5         | 250 ± 0.5           | 0.98 ± 0.2           | 3.9 ± 0.03          |
| 6a           | 0.98 ± 0.1             | 0.49 ± 0.2             | 3.9 ± 0.1           | 0.98 ± 0.4           | 3.9 ± 0.1           | 0.98 ± 0.2           |
| 6b           | 1.95 ± 0.4             | 0.98 ± 0.1             | 62.5 ± 0.3          | 0.98 ± 0.1           | 1.95 ± 0.3           | 3.9 ± 0.03          |
| 8a           | 15.6 ± 0.3             | 125 ± 0.4               | 125 ± 0.3          | 62.5 ± 0.3           | 3.9 ± 0.1           | 62.5 ± 0.5          |
| 9a           | 3.9 ± 0.3              | 1.95 ± 0.08            | 15.63 ± 0.5        | 1.95 ± 0.3           | 250 ± 0.2           | 7.81 ± 0.2           |
| Ciprofloxacin | 0.98 ± 0.2             | 0.1 ± 0.3              | 0.5 ± 0.05         | 0.01 ± 0.1           | -                 | -                 |
| Amphotericin B | -                     | -                      | -                 | -                 | -                 | -                 |

- Not tested; SEM: standard error mean; each value is the mean of three values.
- $^a$Antibacterial and antifungal activities were expressed as MIC in $\mu$g/mL.
Table 2. In vitro inhibitory activities of the screened compounds 3a, 3b, 6a, 6b, 8a and 9a against DHFR enzyme.

| Compound No. | IC_{50} (Mean ± SEM) (μM) |
|--------------|----------------------------|
| 3a           | 0.11 ± 1.05                |
| 3b           | 18.30 ± 0.81               |
| 6a           | 0.09 ± 0.91                |
| 6b           | 5.24 ± 0.37                |
| 8a           | 1.36 ± 0.12                |
| 9a           | 1.45 ± 0.23                |
| Methotrexate | 0.14 ± 1.25                |

IC_{50}: Compound concentration required to inhibit DHFR enzyme activity by 50%; SEM: standard error mean; each value is the mean of three values.

**Structure–activity relationship (SAR) study**

By inspection of the previous data, it was clear that all derivatives having benzene sulphonamide moiety showed higher and remarkable antimicrobial activity than those having napthalenyl one. Incorporation of 3-methyl-5-oxopyrazoline with hydrazinyl open chain at p-4 displayed noticeable and excellent antimicrobial activity especially against *C. albicans* and *A. fumigates* and *C. albicans* in compound 3a. Fusion of the 3-methyl-5-oxopyrazoline with pyridazin moiety giving 6-amino-7-cyano-3-methyl-SH-pyrazolo[4,3-c]pyridazine improved the potency to the highest value in 6a against almost strains in comparison with references. However, 6b revealed approximately the same excellent potency of 3a. Replacement of pyrazidine with triazine or triazole moieties caused drop in the antimicrobial activity in 8a and 9a, respectively.

**In vitro enzyme assay on dihydrofolate reductase (DHFR)**

The inhibitory activity of the synthesised compounds 3a, 3b, 6a, 6b, 8a and 9a were examined against DHFR using reported procedure. The results of the inhibitory activity are shown as IC_{50} values in Table 2 using methotrexate as a positive control. As expressed in Table 2, compounds 3a and 6a proved to be the highest active inhibitors with IC_{50} values 0.11 ± 0.05 and 0.09 ± 0.91 μM, in comparison with Methotrexate (IC_{50} = 0.14 ± 1.25 μM). However, the rest derivatives revealed moderate inhibitory activities via 8a and 9a (IC_{50} = 1.36 ± 0.12 and 1.45 ± 0.23 μM, respectively) and weak ones through 3b and 6b (IC_{50} = 18.30 ± 0.81 and 5.24 ± 0.37 μM, respectively).

Correlation between the chemical structure and the inhibitory activity of the screened derivatives over DHFR enzyme revealed that the existence of benzene sulphonamide moiety in 3a, 6a, 8a and 9a led to enhanced activity, and the potency order was 6a > 3a > 8a > 9a. Moreover, the attachment of pyrazoline ring with hydrazinyl group in 3a or hybridisation with pyridazine in 6a possessed better inhibitory activity than with triazine or triazole in 8a and 9a.

**In silico calculations of molecular properties**

**Drug-likeness parameters**

A molecular property is a complex balance of various structural features which determine whether a specific molecule is similar to the known drugs and to facilitate the drug-likeness of the candidate drug. Therefore, the calculated molecular descriptors of the new compounds expressed in terms of calculating log P, molecular size, flexibility and the presence of hydrogen-donor and acceptors using Molinspiration tool and the results are depicted in Table 3.

By analysing the obtained data, it can be concluded that there are linear correlations between the lipophilicity and the activity of the new derivatives and all of them are fully in agreement with the Lipinski’s rule. All the tested compounds have the number of rotatable bonds in the range of 1–3, with the values ranging from 2 into 3 allowed to the most bioactive compounds 3a, 3b, 6a, 6b, 8a and 9a and therefore, obviously exhibiting small conformational flexibility. Results shown in Table 3 indicated that all of the analysed compounds have Topological polar surface area (TPSA) values < 140 Å² except for compounds 6a and 6b have values 153.59 and 147.54, respectively, and therefore are candidate for good solubility, capacity for penetrating cell membranes and intestinal absorption. Compounds with logP value (H-bond donors) less than 5 exhibited increased solubility in cellular membranes and all the test compounds have logP value in the range of 0–4 which is less than 5. Compounds have logP (H-bond acceptors) in the range of 5–9 and molecular weight in the range of 249.28–398.47. Molecular volumes of the compounds in the series increased as increasing MW and ranged from 216.61 to 317.53. The partition coefficient is vital to examine the physico-chemical properties associated with biological activity. The miliogP values for all compounds under investigation are less than 5, outlining good lipophilicity (as defined by the pH-partition hypothesis) with various possible biological sites reasonable oral absorption and lower permeation across biological membranes but higher aqueous solubility especially for compounds 3a, 6a, 6b, 8a and 9a that are most bioactive compounds. Higher solubility is a good factor in drug formation and might generally be reduced in more highly hydrophobic compounds. The greater lipophilicity of these compounds may protect against ROS damage and is due in part to their smaller polar surface area which is another useful descriptor of the oral bioavailability and drug transport properties. As can be seen, compounds 3b and 6c that contains the nonpolar napthalenyl substituent exhibited greater lipophilicity (miliogP = 2.54 and 2.63,
respectively) as compared to compound 3a and 6a (milogP = 0.07 and −0.04) with polar group (sulphonamide). Also, the same phenomena were observed in compounds 6d, 6b, 9b, that contain naphthyl substituent exhibited greater lipophilicity (milogP = 2.48, 3.94, 2.70, respectively) as compared to compounds 6b, 8a, 9a (milogP = −0.02, 1.27, 0.03) with polar group (sulphonamide). Our study revealed that compounds 6c, 6d, 8a, 9b (greater lipophilicity) are biologically either inactive or less active, but compounds 6a, 6b, 8a and 9a (least lipophilicity) are highly active against the tested bacteria and fungi, while compound 3a are highly active than 3b. Our results exhibited that there is a clear relationship between lipophilicity and antimicrobial activity. The most active compounds against all microbial strains were 3a (milogP = 0.07), 6a (milogP = −0.04), 6b (milogP = −0.02), 8a (milogP = 1.27) and 9a (milogP = 0.03). In the light of the above results, we achieved that all tested compounds satisfy the “Rule of five” and meet all criteria for good permeability and bioavailability.

**Toxicity risks**

Toxicity risks and physicochemical properties of the newly prepared compounds were evaluated through the methodology developed by Osiris. The data given in Table 4, displayed that all compounds are expected to be non-irritating and high risk of reproductive effects. Also, compounds 3b, 6b, 6d, 8b and 9b have shown the non-mutagenic and non-tumorigenic effects. The aqueous solubility of a compound significantly affects its absorption and distribution properties. It is well known that more than 80% of the drugs on the market have estimated solubility values greater than −4. From Table 4, it was noted that compounds 3a, 3b, 6b, 9a and 9b exhibited solubility values above −4 and they are expected to have good aqueous solubility which significantly affects their absorption and distribution characteristics. Osiris programme was used for calculating the fragment-based drug-likeness of the synthesised compounds; a positive value indicates that the designed molecule contains fragments that are frequently present in commercial drugs. The results indicated that all target compounds have drug-likeness values except 6c and 6d in the comparable zone with that of the standard drugs. The drug score combines drug likeness, milogP, solubility, molecular weight, and toxicity risks in one handy value that may be used to judge the compound’s overall potential to qualify for a drug. A value of 0.5 or more makes the compound a promising lead for future development of safe and efficient drugs. The overall drug score values for the synthesised compounds were calculated and compared to those of the standard drugs. Compounds 3a, 6b and 9a own good drug score values (Table 4).

### Table 3. Calculated molecular properties of the synthesised compounds 3, 6, 8 and 9 for assessment of the drug likeness.

| Compound No. | Rule | mLogP<sub>6</sub> | % ABS<sup>b</sup> | TPSA<sup>c</sup> | N<sub>Atoms</sub><sup>d</sup> | MW<sup>e</sup> | M.Vol. | n<sub>NOn</sub> | n<sub>Viol</sub> | n<sub>Tot</sub><sup>f</sup> |
|--------------|------|-----------------|-----------------|-----------------|-----------------|-------------|---------|----------------|----------------|-----------------|
| 3a           | 0.07 | 64.04           | 130.31          | 19              | 281.30          | 222.79      | 8       | 4              | 0              | 3               |
| 3b           | 2.54 | 84.80           | 70.14           | 19              | 252.28          | 224.06      | 5       | 2              | 0              | 2               |
| 6a           | −0.04| 56.01           | 153.59          | 23              | 329.35          | 259.77      | 9       | 4              | 0              | 2               |
| 6b           | −0.20| 58.09           | 147.54          | 23              | 330.33          | 256.61      | 9       | 3              | 0              | 2               |
| 6c           | 2.63 | 76.77           | 93.42           | 23              | 300.32          | 261.04      | 6       | 2              | 0              | 1               |
| 6d           | 2.48 | 78.85           | 87.37           | 23              | 301.31          | 257.88      | 6       | 1              | 0              | 1               |
| 8a           | 1.27 | 71.49           | 108.71          | 27              | 398.47          | 316.26      | 8       | 2              | 0              | 3               |
| 8b           | 3.94 | 92.25           | 48.54           | 27              | 369.45          | 317.53      | 5       | 0              | 0              | 2               |
| 9a           | 0.03 | 67.74           | 119.57          | 19              | 278.30          | 216.61      | 8       | 3              | 0              | 2               |
| 9b           | 2.70 | 88.50           | 59.40           | 19              | 249.28          | 217.88      | 5       | 1              | 0              | 1               |
| Ciprofloxacin| −0.70| 83.27           | 74.57           | 24              | 331.35          | 285.46      | 6       | 2              | 0              | 3               |
| Amphotericin B| −2.49| 1.27            | 319.61          | 65              | 924.09          | 865.48      | 18      | 13             | 3              | 3               |

*Octanol–water partition coefficient, calculated by the methodology developed by Molinspiration.

% ABS: percentage of absorption.

TPSA: topological polar surface area.

Number of non-hydrogen atoms.

Molecular weight.

Molecular volume.

Number of hydrogen-bond acceptors (O and N atoms).

Number of hydrogen-bond donors (OH and NH groups).

Number of “Rule of five” violations.

Number of rotatable bonds.

### Table 4. Toxicity risks, solubility, drug-likeness, and drug score of the synthesised compounds.

| Comp. no. | Toxicity risks | Solubility | Drug likeness | Drug Score |
|-----------|----------------|------------|---------------|------------|
| 3a        | green green green red | −2.09 | 6.32 | 0.56 |
| 3b        | red red green red | −3.8 | 0.81 | 0.17 |
| 6a        | green green green green | −4.15 | 0.33 | 0.38 |
| 6b        | green green green red | −2.79 | 1.59 | 0.49 |
| 6c        | red red green red | −5.86 | −1.42 | 0.07 |
| 6d        | red red green red | −4.5 | −0.01 | 0.12 |
| 8a        | red red green green | −4.4 | 0.47 | 0.24 |
| 8b        | red red green red | −6.11 | 3.16 | 0.08 |
| 9a        | green green green red | −2.22 | 4.26 | 0.56 |
| 9b        | red red green green | −3.93 | 2.52 | 0.17 |
| Ciprofloxacin | green green green green | −3.32 | 2.07 | 0.82 |
| Amphotericin B | green green green green | −5.08 | −0.14 | 0.27 |

Red: high risk; green: low risk.
Molecular modelling study

In the current study, the molecular docking study was performed in order to rationalise the obtained *in vitro* enzyme assay results on dihydrofolate reductase. So, the interactions of the highly potent synthesised compounds 3a and 6a with the active site of DHFR were explored using MOE (Molecular Operating Environment) software 10.2008,60,61 and through downloading of the protein data bank file (PDB ID: 1DLS)62 that contains the co-crystallised ligand, methotrexate. The molecular docking protocol was verified by re-docking of the original ligand in the vicinity of the active site of DHFR indicating that the docking protocol used is suitable for the intended docking study. This is shown by the score energy of −11.68 kcal/mol, the small root mean standard deviation (RMSD) between the experimental co-crystallised inhibitor pose and the docked pose of 0.88 Å and the highly noticed superimposition between them (Figure 5(c)).

The pteridine moiety of the co-crystallised ligand, methotrexate interacts with the active site of DHFR by two different interactions52; hydrogen bonding of the two amino groups with the side chain of Ser59 (distance: 2.85 and 3.05 Å) (Figure 6). The oxygen of pyrazolone displays two H-bonds, acceptor with the side chain of Ser59 (distance: 2.64 Å), while the two oxygen of the carboxylic group shares the binding by two H-bond acceptors with Arg70 (distance: 2.68 and 3.12 Å, respectively). This beside many hydrophobic interactions with various amino acid residues: Ile7, Val8, Ala9, Tyr22, Arg28, Phe31, Gln35, Ser59, Ile60, Pro61, Val115, Tyr121, and Thr136, as shown in (Figure 5).

The docked compounds 3a and 6a explored higher negative energy score of −12.17 and −13.48 kcal/mol indicating higher predicted binding affinity than the co-crystallised ligand. They were fit in the active site of the enzyme in a similar way via binding of the sulphonamide moiety with the side chain of Ser59 through two hydrogen bonds (Figures 6 and 7). The two nitrogen of the hydrazinyl moiety in 3a displayed two H-bonds, acceptor with the backbone of Ser118 and donor with side chain of Thr146 (distance: 2.72 and 1.62 Å, respectively). The oxygen of pyrazolone scaffold shared fixation through two H-bond acceptors with the side chain of Ser119 (distance: 2.85 and 3.05 Å) (Figure 6).

The bulky pyrazol(4,3-c)pyridazine scaffold in the compound 6a enforced a bound conformation via two arene–arene interactions with Phe31. It could be assumed that chain elongation with −NH–N= hydrazinyl fragment between pyrazole and benzenesulfonyamide in 3a, deprived pyrazole ring from binding with the essential amino acid Phe31, although the insertion of N–N chain in cyclised ring fused to pyrazole in 6a gave the chance to be near with Phe31. Furthermore, the cyano substitution on the pyrazolopyridazine moiety in 6a allowed two hydrogen bond acceptors with the side chain of Arg28 (distance: 2.71 and 2.92 Å, respectively) (Figure 7).

Finally, the presence of the sulphonamide substitution in the examined compounds allowed for the H-bond formation with the side chain of Ser59 that was responsible for the observed superior inhibitory activities against DHFR. Notably, the 7-cyanopyrazol(4,3-c)pyridazine substitution demonstrated excellent binding profile leading to synergistic effect.

Conclusion

In summary, a series of pyrazole derivatives 3, 6, 8 and 9 bearing different heterocyclic systems was designed and synthesised. All synthesised analogues were examined for their *in vitro* antimicrobial, DHFR inhibition activity and *in silico* studies. The antimicrobial data revealed the ability of the compounds 3a and 6a to inhibit the growth of the screened panel of six strains with excellent MIC values in comparison with the reference drugs. In addition, the *in vitro* inhibitory activity against DHFR enzyme illustrated that compounds 3a and 6a were the most potent ones in comparison with methotrexate. Based on the previously obtained data from DHFR inhibition assay, the pyrazoles 3a and 6a containing sulphonamide substitution illustrated good fitting and favourable binding modes with DHFR enzyme in the docking study through formation of a critical hydrogen bond with the essential amino acid Ser59.

Disclosure statement

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