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***Vitex Negundo*–Fe₃O₄–CuO green nanocatalyst (VN–Fe₃O₄–CuO): synthesis of pyrazolo[3,4-*c*]pyrazole derivatives via the cyclization of isoniazid with pyrazole and their antimicrobial activity, cytotoxicity, and molecular docking studies†**

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In this study, we developed a novel pyrazolo[3,4-*c*]pyrazole derivative with antibacterial and antifungal activities that shows great potential for treating infectious diseases. To evaluate the binding affinity of 1AJ0 and 1AI9 proteins for developing potent antibacterial and antifungal compounds, we used the *Vitex negundo* (VN) leaf extract as the capping and reducing agent and reacted it with Fe₂O₃ and Cu(OAc)₂ solutions to synthesize the VN–Fe₃O₄–CuO nanocatalyst. The newly synthesized compounds were confirmed using Fourier transform infrared spectroscopy, transmission electron microscopy, UV-visible spectroscopy, and X-ray diffraction analyses. Antibacterial screening revealed that compound **1g** was highly active against *Escherichia coli* (MIC: 1 µg mL^{−1}) and was much more effective than the standard ciprofloxacin. Compound **1b** showed a higher antifungal activity than clotrimazole against *Candida albicans* (MIC: 0.25 µg mL^{−1}) and cytotoxic activity against MCF-7 cancer cell lines. Compounds **1a–1l** were exhibited low cytotoxicity activity compared to the standard doxorubicin (LC₅₀: 21.05 ± 0.82 µg mL^{−1}). To further support the discovery of new active antibacterial agents, compounds **1g** and **1b** and proteins 1AJ0 and 1AI9 were examined using the AutoDock Vina program and were compared with the standards ciprofloxacin and clotrimazole. With the 1AJ0 protein, compound **1g** had a higher docking score (−3.7 kcal mol^{−1}) than ciprofloxacin (−5.6 kcal mol^{−1}), and with the 1AI9 protein, compound **1b** had a higher docking score (−4.8 kcal mol^{−1}) than clotrimazole (−4.4 kcal mol^{−1}). Additionally, molecular dynamics simulation was used to investigate the most probable binding mode of compounds **1b** and **1g** with 1AI9 and 1AJ0, respectively. The VN–Fe₃O₄–CuO catalyst was used to prepare pyrazolo[3,4-*c*]pyrazole derivatives, which were successfully characterized and screened for antimicrobial and cytotoxic activities, molecular docking, and molecular dynamics simulation studies.

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1. Introduction

Pyrazolo[4,3-*c*]pyrazoles derivatives are an intriguing class of compounds that act as isosteric purine derivatives with potential pharmacological effects. They are also useful intermediates and end products in the synthesis of physiologically and

pharmacologically active compounds, such as dyes and antibiotics, that contain pyrazoles.^{1–3} The ATP (adenosine triphosphate)-binding site of protein kinases is the target for a family of substances known as tetrahydropyrrolo[3,4-*c*]pyrazoles.⁴ Pyrazoles derivatives contain N–N bonds and are found in five-membered heterocycles,⁵ and are commonly applied in biochemical and pharmaceutical fields (Fig. 1).⁶

Monocyclic pyrazoles can serve as synthetic scaffolds for fused heterocyclic systems, including pyrazolo-fused pyrimidines, quinolones, pyridines, thiazoles, isoquinolines, imidazoles, diazepines, and triazines.⁷ They have potent biological activities, including immunostimulatory, anticancer, antioxidant, antibacterial, and antiviral effects.⁸

Some bicyclic heterocycles are effective hGSK-3α inhibitors and can treat esophageal and gastrointestinal mucosal injuries.⁹ Pyrazolo[3,4-*c*]pyrazoles derivatives are used to treat autoimmune and inflammatory diseases because of their diverse

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† Electronic supplementary information (ESI) available: The experimental procedures for the cytotoxic activity of compounds (**1a–1l**), molecular dynamics simulation studies, and NMR studies. See DOI: <https://doi.org/10.1039/d3ra06771h>



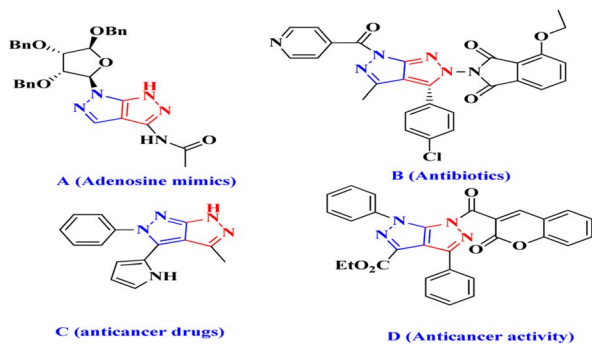
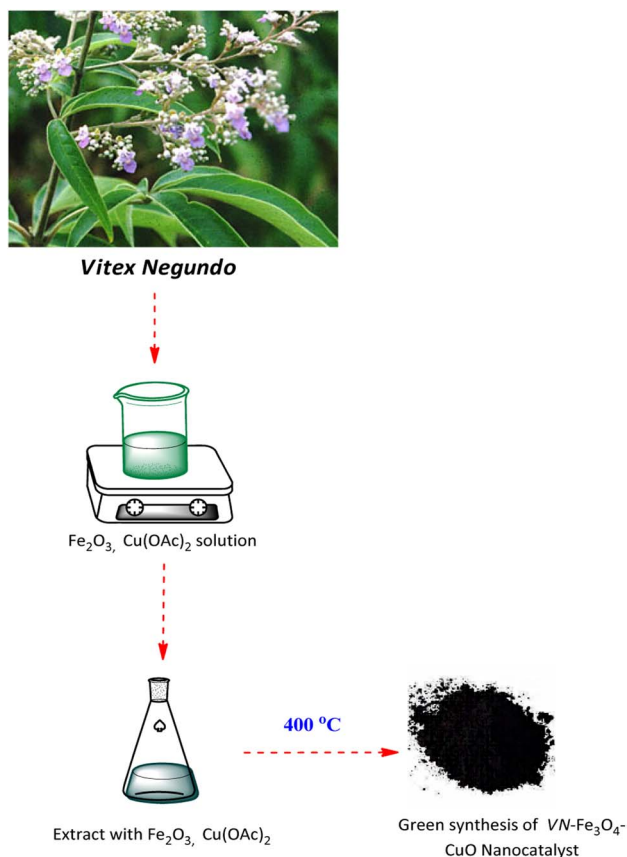


Fig. 1 Bioactive pyrazolo[3,4-c]pyrazoles derivatives.



Scheme 1 Synthesis of the VN-Fe₃O₄-CuO nanocatalyst.

pharmacological and biological properties. Adenosine mimics,¹⁰ antibiotics,¹¹ and anticancer drugs¹² are examples of compounds that inhibit liver cancer growth in HepG2 cells (anticancer activity) (D)¹³ (Fig. 1). Additionally, various medications have been shown to contain pyrazolo[3,4-c]pyrazole derivatives, which is a physiologically active molecule.¹⁴ Previously documented techniques include the reaction of 4-arylidene-pyrazol-5-ones with hydrazine and hydrazide to produce physiologically active substituted pyrazolo[3,4-c] pyrazoles derivatives.¹⁵ Nanotechnology involves applying structures at the nanoscale (1–100 nm) by controlling their size and shape, specifically using nanocatalysts.¹⁶ The

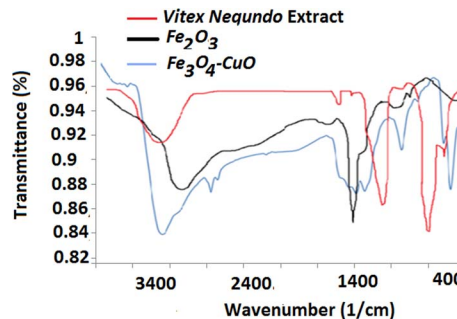


Fig. 2 FTIR spectrum of the VN-Fe₃O₄-CuO nanocatalyst.

synthesis of nanomaterials using plant extracts, specifically including Ag,¹⁷ Au,¹⁸ Cu,¹⁹ Pd,²⁰ CuO,²¹ MgO,²² ZnO,²³ and Cu catalysts²⁴ is an example of green synthesis. Metal nanocatalysts are inexpensive, environment-friendly, size-dependent, and exhibit desirable electrical properties.²⁵ *Vitex Negundo* (VN) is an effective plant used in South Asia, China, East Africa, Japan, Indonesia, and South America.²⁶ It belongs to the Verbenaceae family and comprises 250 species. VN contains anticancer, anti-fungal, antibacterial, and anti-inflammatory compounds.²⁷ There is a high demand for nanocatalysts due to their adaptability and outstanding biological, catalytic, and atomic activities.²⁸ This study focused on analysing the VN-Fe₃O₄-CuO nanocatalyst because it represents the first comprehensive investigation of the use of VN powder extract VN-Fe₃O₄-CuO nanocatalyst for the synthesis of pyrazolo[3,4-c] pyrazole derivatives, which exhibited potent antimicrobial and cytotoxic activities. Molecular docking and molecular dynamics simulations were performed to further investigate the underlying mechanisms of these activities.

2. Materials and methods

2.1. Reagents and materials

¹H and ¹³C NMR analyses were carried out using Bruker DRX-300 MHz and 75 MHz spectrometers. FT-IR (KBr) spectra, ranging from 4000–400 cm⁻¹, were obtained on a Nicolet iS5 instrument from Thermo Scientific. The silica gel plates were purified using thin-layer chromatography. A Varioel III elemental analyser was used to calculate the percentages (%) of C, H, S, and N. Mass spectra were recorded on a Clarus 690 – SQ8MS (EI) instrument from PerkinElmer GCMS.

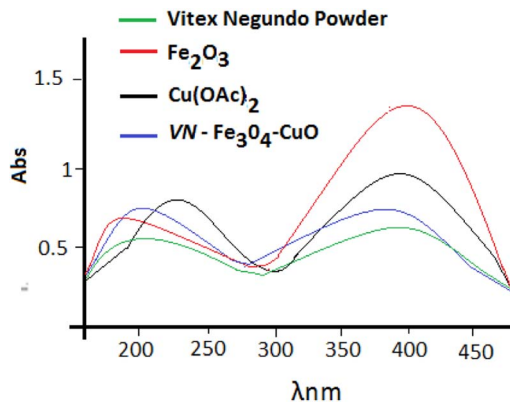
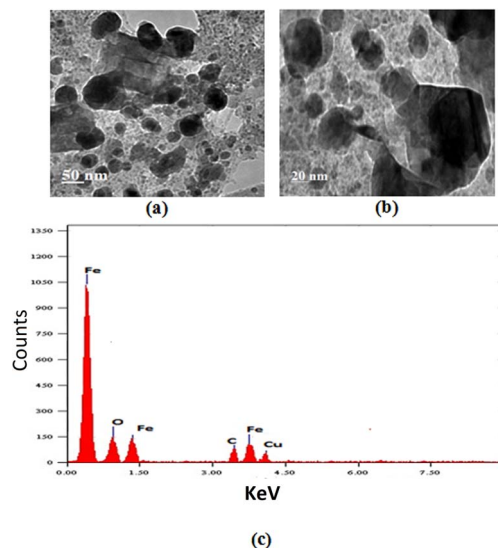
2.2. Preparation of the Vitex Negundo powder

Crushed *Vitex Negundo* leaves were sieved through 20# mesh after being crushed into a fine powder. To prepare the leaf extract, 200 mL of double-distilled water was boiled with 5 g of powdered leaves for 10 min. The filtered broth was stored at 41 °C after it had been filtered through a 0.2 mm filter for a week. The synthesis of VN-Fe₃O₄-CuO nanocatalyst is outlined in Scheme 1.

2.3. The synthesis of VN-Fe3O4-CuO nanocatalyst

The VN powder extract (50 mL) was added to a mixed aqueous solution containing 1.0 mmol Cu(OAc)₂, and 1.0 mmol iron(III)

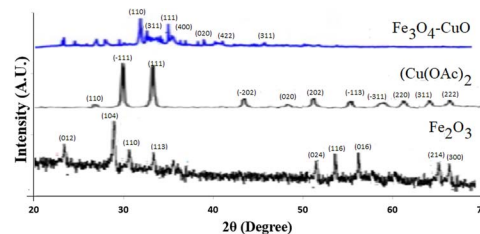


Fig. 3 UV-vis spectra of the VN-Fe₃O₄-CuO nanocatalyst.Fig. 4 TEM image for VN-Fe₃O₄-CuO nanocatalyst: (a) 50 nm and (b) 20 nm, and (c) an EDAX of the VN-Fe₃O₄-CuO nanocatalyst.

oxide. Following the addition of the plant extract, bioreduction occurred, resulting in the formation of the Fe₃O₄. The VN-Fe₃O₄-CuO nanocatalyst was purified by centrifugation at 10 000 rpm for 20 min. The mixture was refluxed for 1 h, during which the solution's original colour changed to dark brown, indicating the formation of the VN-Fe₃O₄-Cu nanocatalyst. The resulting nanocatalyst was then dried at 40 °C and then converted into the VN-Fe₃O₄-CuO nanocatalyst.

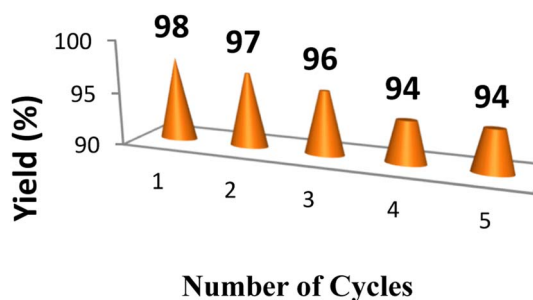
2.4. Characterization of the synthesized VN-Fe₃O₄-CuO nanocatalyst and pyrazolo[3,4-*c*]pyrazole derivatives

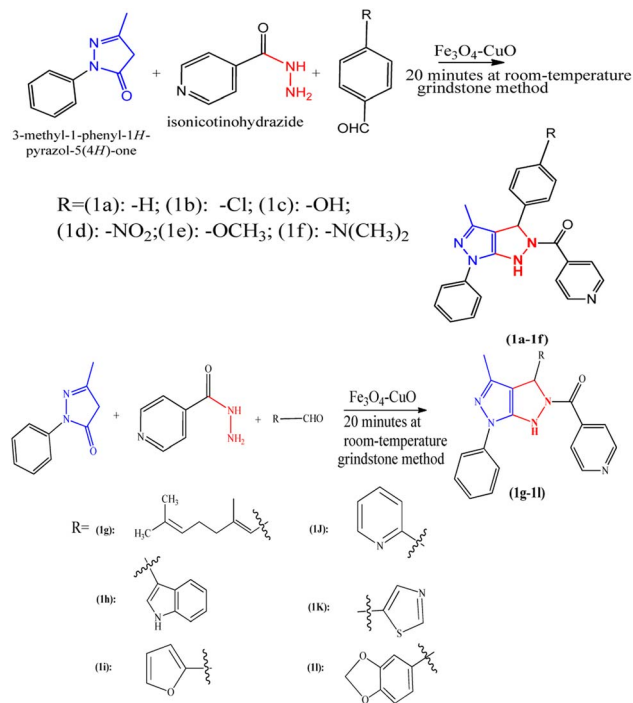
A spectrophotometer was used to monitor the bioreduction of iron salt, and a quartz cuvette was used as the standard. The UV-vis spectrum of VN-Fe₃O₄-CuO nanocatalyst was recorded in the range of 200 to 600 nm. We used a Nicolet iS5 FTIR spectrometer (4000–400 cm⁻¹) obtained from Thermo Scientific to record the IR spectra of the VN powder extract, Fe₃O₄, and VN-Fe₃O₄-CuO nanocatalyst. The VN-Fe₃O₄-CuO nanocatalyst was examined

Fig. 5 XRD patterns of the VN-Fe₃O₄-CuO nanocatalyst.

using TEM (JEOL, JAM-1011) to obtain a VN-Fe₃O₄-CuO nanocatalyst of approximate size of 50 nm. The VN-Fe₃O₄-CuO nanocatalyst was poured onto the grids made of copper that had been coated with carbon during the evaporation phase and left to evaporate in the open air. The resolution of the TEM was 20 nm, and the length was 1.2 mm. It was operated between 40 and 100 keV. An ultrathin windowed liquid-nitrogen-cooled EDX was used to analyze the chemical makeup of the VN-Fe₃O₄-CuO nanocatalyst. XRD, (Ultima IV) was used to analyze the VN-Fe₃O₄-CuO nanocatalyst. After the nanocatalyst had been dried in a vacuum oven at 40 °C, the powder was manually grind. The VN-Fe₃O₄-CuO nanocatalyst exhibited a series of characteristic peaks at 34.05, 34.07, 36.11, 37.24, 40.04, 43.38, and 47.282.

2.4.1. Synthesis of (3-(4-chlorophenyl)-4-methyl-6-phenylpyrazolo[3,4-*c*]pyrazol-2(1*H*,3*H*,6*H*)-yl)(pyridin-4-yl)methanone 1a. The VN-Fe₃O₄-CuO nanocatalyst (30 mol%) was added to a mixture containing 3-methyl-1-phenyl-1*H*-pyrazol-5(4*H*)-one (0.01 mol), benzaldehyde (0.01 mol), and isonicotinohydrazide (0.01 mol) in ethanol (10 mL) and the reaction mixture was refluxed for 30 min. The progress of the reaction was monitored using thin-layer chromatography. After completion of the reaction, the catalyst was filtered by hot water washing, and the filtered solution was extracted with ethyl acetate (10 mL × 3) and washed with saturated sodium bicarbonate (50 mL) and brine (30 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to obtain the crude residue, which was purified by column chromatography on silica gel using hexane/EtOAc (4:6, v/v) to produce the desired product. The above method was followed to synthesize other compounds (**1b–1l**). Physical constants, spectral data, molecular mass, and analytical values of compounds (**1a–1l**) are provided in the ESI files.†

Fig. 6 Recycling of the VN-Fe₃O₄-CuO nanocatalyst for the synthesis of compound 1a.



Scheme 2 The synthetic route of pyrazolo[3,4-c]pyrazole derivatives.

2.5. Recyclability study

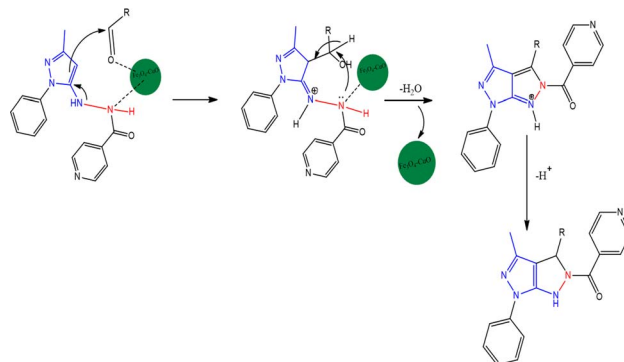
The stability and catalytic activity of the VN- $\text{Fe}_3\text{O}_4\text{-CuO}$ nano-catalyst synthesized using the green method was assessed through a recycling experiment. Using a conventional approach, the reaction was performed as previously described. Following the reaction, the catalyst was filtered, washed with hot water, and dried for 3 h at 80 °C in a vacuum oven before the next cycle, as shown in (Fig. 6).

2.6. Biological activity

The antimicrobial activity of the concentrated extracts was tested on nine bacterial strains, including both Gram-positive and Gram-negative strains, which were obtained from the Microbial Type Culture Collection (MTCC) located at the Institute of Microbial Technology in Chandigarh, India. (See ESI† for full details).

2.6.1. Antibacterial activity. The disk diffusion method was used to evaluate the activity of each compound.²⁹ Gram-positive bacteria were *Enterococcus Faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC 96), and other bacteria whereas Gram-negative bacteria examined were *Pseudomonas aeruginosa* (MTCC 739), *Klebsiella pneumonia* (MTCC 109), and *Escherichia coli* (MTCC 739). DMSO was used to dissolve each test sample at a concentration of 100 $\mu\text{g mL}^{-1}$. Inhibition was determined following a 24-hour incubation period at 37 °C. Ciprofloxacin was used as the test compound.

2.6.2. Antifungal activity. All compounds were subjected to agar dilution testing to assess their antifungal activity.²⁹ The compounds (1a-1l), *Candida albicans* (MTCC 183), *Microsporum audouinii* (MTCC 739), *Cryptococcus neoformans* (a clinical



Scheme 3 The possible mechanism for the synthesis of pyrazolo[3,4-c]pyrazole derivatives.

isolate), and *Aspergillus niger* (MTCC 872), were used in this study. DMSO (100 $\mu\text{g mL}^{-1}$) was used to determine the sample concentration. Clotrimazole was the standard for comparison of the test compounds.

2.6.3. Minimum inhibitory concentration (MIC) studies. To determine the minimal inhibitory concentrations (MICs) of compounds (1a-1l), their 64 $\mu\text{g mL}^{-1}$ solutions were subjected to successive two-fold dilutions, furnishing samples with concentrations of 64, 32, ..., and 0.25 $\mu\text{g mL}^{-1}$. Microbial suspensions of 106 CFU mL^{-1} (CFU = colony forming unit) were inoculated in the corresponding wells, and the plates were incubated at 36 °C for 24 h. The MICs were determined as the lowest concentrations completely inhibiting the visible growth of microorganisms.

2.6.4. Cell lines and cell cultures. The American-type cell collection produced MCF-7 cell lines; the cell lines and cell culture details are available in the ESI.†

2.6.5. Cytotoxic activity. The cytotoxicity of the synthesized compounds (1a-1l) was assessed following the experimental procedure discussed previously.^{30,31}

2.7. Molecular docking

2.7.1. Preparation of ligands. ChemDraw 12.0 was used to draw the ligand molecules (1a-1l), and Chem3Dpro's MM2 force field was used to minimise the energy.

2.7.2. Preparation of receptor. The ternary complex of *Escherichia coli* and *Candida albicans* and proteins' 3D of the Protein Data bank's proteins' 3D crystal structures were downloaded using PDB IDs 1AI9 and 1AJ0. Discovery Studio 2019 was used to remove water molecules and ligands from the receptor. The SWISS PDB Viewer software was utilized to minimize the energy consumption of the receptor during the molecular docking analysis.

2.7.3. Identification of binding pocket. Discovery Studio 2019 was used for the identification of the binding pockets of the target protein that contains Phe 36, Asn 22, Thr 62, Arg 63, Arg 255, Arg 63, Arg 255, and Ile 257 residues.

2.7.4. Docking. The interactions of the binding modes between compounds (1a-1l), 1AI9, and 1AJ0 proteins were examined using the Auto Dock Vina 1.1.2 program for



Table 1 Optimization study of the reaction conditions for **1a**

S. no.	Catalysis	Reaction condition, RM, grindstone method	Mol percentage of catalysis	Yield
1	CuO	1 min	10	—
			20	—
			30	—
		10 min	10	—
			20	—
			30	—
		20 min	10	—
			20	—
			30	10
		30 min	10	—
			20	—
			30	23
2	Cu(OAc) ₂	1 min	10	—
			20	—
			30	—
		10 min	10	—
			20	12
			30	14
		20 min	10	—
			20	15
			30	17
		30 min	10	—
			20	13
			30	26
3	Fe ₂ O ₃	1 min	10	—
			20	—
			30	—
		10 min	10	—
			20	—
			30	12
		20 min	10	—
			20	12
			30	24
		30 min	10	—
			20	15
			30	32
4	Fe ₃ O ₄	1 min	10	—
			20	—
			30	—
		10 min	10	—
			20	—
			30	14
		20 min	10	—
			20	—
			30	12
		30 min	10	—
			20	—
			30	17
5	VN-Fe ₃ O ₄ -CuO	1 min	10	—
			20	10
			30	15
		10 min	10	37
			20	48
			30	82
		20 min	10	63
			20	81
			30	94
		30 min	10	89
			20	92
			30	98

Table 2 The minimal inhibitory concentrations of the compounds (1a–1l)^a

Com. no.	Antibacterial activity					Antifungal activity			
	Gram-negative bacteria			Gram-positive bacteria					
	<i>E. c</i>	<i>P. a</i>	<i>K. p</i>	<i>S. a</i>	<i>E. f</i>	<i>A. n</i>	<i>C. a</i>	<i>C. n</i>	<i>M. a</i>
1a	64	64	32	>100	>100	64	64	>100	>100
1b	>100	>100	4	>100	8	8	0.25	32	>100
1c	16	32	>100	>100	16	>100	64	>100	64
1d	32	64	>100	64	>100	>100	>100	64	32
1e	32	>100	>100	64	32	64	16	>100	1
1f	>100	16	8	8	>100	>100	32	>100	>100
1g	1	32	64	>100	64	32	32	64	64
1h	>100	32	>100	>100	>100	64	32	4	0.5
1i	64	>100	16	64	16	>100	>100	2	64
1g	>100	>100	32	>100	32	16	64	16	>100
1k	64	>100	32	32	>100	32	>100	64	>100
1l	>100	>100	>100	>100	>100	>100	>100	64	64
DMSO	–	–	–	–	–	–	–	–	–
(a) ^b	32	64	32	0.12	16	–	–	–	–
(b) ^b	–	–	–	–	–	1	0.5	4	2

^a The minus (–) represents inactive for screening. ^b Standard (a): ciprofloxacin; standard (b): clotrimazole.

molecular docking. The active amino acid residues in the binding pocket formed a docking grid box. The Pymol and Discovery Studio 2019 programming were used to visually analyse the interactions with the exhaustiveness value set to 8.^{32–34}

2.7.5. Molecular dynamics simulation. Molecular dynamics simulation was conducted on the docked complexes identified through IFD analysis, utilising Desmond of Schrödinger Biosuite, to evaluate their stability.³⁵

3. Results and discussion

3.1. Characterization of the VN-Fe₃O₄-CuO nanocatalyst

Identification of *Vitex Negundo* leaf extracts. Characteristic peaks for the hydroxyl compounds O–H (stretch) were obtained in the aqueous solution (3276 cm^{–1}). N–H (stretch), and C–N (stretch) characteristic peaks, in the aqueous extract at 1169 cm^{–1} and 761 cm^{–1}.³⁶ The synthesized Fe₂O₃ exhibited a large broad band at 3396 cm^{–1}, which is ascribed to the O–H stretching vibration in the O–H groups. The peak at 1482 cm^{–1} is attributed to the symmetric bending vibration of C=O.³⁷ The bands of the VN-Fe₃O₄-CuO nanocatalyst were observed at 3400 and 1271 cm^{–1}. The presence of the O–H stretching vibration was indicated by the band at 3400 cm^{–1} in the *Vitex Negundo* leaf extract.³⁸ Peaks at 752 and 591 cm^{–1} were utilized to demonstrate the development of CuO nanostructures as reducing and capping agents for the synthesis of the VN-Fe₃O₄-CuO nanocatalyst³⁹ (Fig. 2).

The VN-Fe₃O₄-CuO nanocatalyst was studied using UV-vis spectroscopy. The reaction mixture of the leaf extract and Fe₃O₄ solution immediately turned black because of the formation of the VN-Fe₃O₄-CuO nanocatalyst. Plant extracts contain bioactive molecules with hydroxyl groups that reduce

iron to ferric hydroxide, which is partially converted into the VN-Fe₃O₄-CuO nanocatalyst by other bioactive compounds. In the visible region, the VN-Fe₃O₄-CuO nanocatalyst exhibited significant absorption at 242 nm, Fe₂O₃ absorption peak at 387 nm, and Cu(OAc)₂ absorption peak at 290, and the VN-Fe₃O₄-CuO nanocatalyst absorption peak changed slightly towards 230 nm, indicating that the VN-Fe₃O₄-CuO nanocatalyst 275 nm was formed (Fig. 3).

The solutions of the VN-Fe₃O₄-CuO nanocatalyst were spread over a carbon-coated grid at room temperature for TEM measurements. Fig. 4a and b show TEM images of the VN-Fe₃O₄-CuO nanocatalyst that were mostly spherical and 20–50 nm in size. The particle size distribution histogram was

Table 3 Cytotoxic activity of compounds (1a–1l)

Compounds	LC ₅₀ ^a (μg mL ^{–1})		
	MCF-7 cell line	Vero	SI ^b
1a	78.10 ± 0.02	55.02 ± 0.11	0.70
1b	22.10 ± 0.04	65.23 ± 0.05	2.95
1c	70.10 ± 0.05	73.81 ± 0.07	1.05
1d	91.10 ± 0.06	83.36 ± 0.03	0.91
1e	23.62 ± 0.02	79.39 ± 0.09	3.36
1f	19.92 ± 0.01	82.10 ± 0.07	4.12
1g	30.82 ± 0.04	65.66 ± 0.06	4.11
1h	21.43 ± 0.03	88.10 ± 0.05	2.75
1i	26.66 ± 0.05	73.45 ± 0.01	3.55
1j	57.34 ± 0.08	83.20 ± 0.02	1.45
1k	62.34 ± 0.07	78.01 ± 0.05	1.25
1l	69.12 ± 0.11	88.50 ± 0.02	1.28
Doxorubicin	0.65 ± 0.01	21.05 ± 0.82	32.38

^a The mean values of three separate experiments are represented as mean ± standard error. ^b Selectivity index = LC₅₀ value normal cell/LC₅₀ value cancer cell.



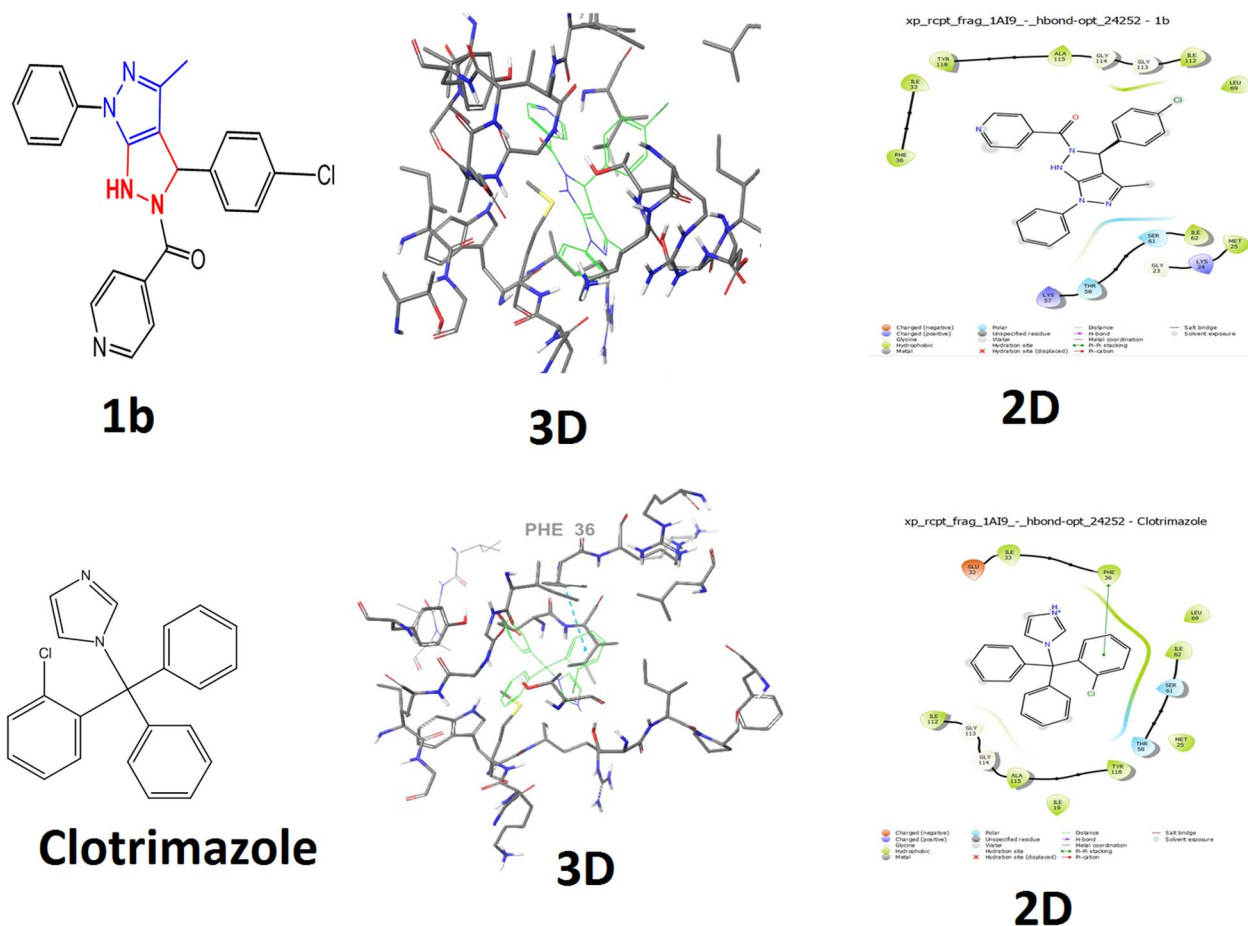


Fig. 7 Molecular docking studies for protein IAI9 (*Candida albicans*) docked with compound **1b** and clotrimazole with their 2D and 3D docking structures.

determined based on the sizes of 82 particles from the TEM image. The majority of the spherical nanocatalyst were single crystals. A characteristic optical absorption peak was observed at 0.5 keV, and EDAX detected Fe and oxygen, indicating the formation of the VN-Fe₃O₄-CuO nanocatalyst, as shown in Fig. 4c. The XRD patterns of the VN-Fe₃O₄-CuO nanocatalyst matched the (110), (311), (111), (400), (020), (422), and (311) Bragg reflections corresponding to the characteristic peaks at 34.05, 34.07, 36.11, 37.24, 40.04, 43.38, and 47.28, respectively. The Cu(OAc)₂ patterns matched the (110), (111), (311), (400), and (422) Bragg reflections corresponding to the characteristic peaks at 27.00, 31.14, 38.21, 44.06, 47.17, 51.24, 56.31, 59.28, 63.34, 65.07, and 67.032, respectively.

Each of these patterns matched the (110), (−111), (111), (−202), (020), (−113), (−311), (220), (311), and (222) Bragg reflections corresponding to the characteristic XRD peaks as 24.06, 28.12, 31.46, 34.72, 51.04, 54.16, 57.31, 65.28, and 56.28 2 for Fe₂O₃. These patterns were matched to the (012), (104), (110), (113), (024), (116), (0.16), (214), and (300) Bragg reflections according to the JCPDS standard magnetite XRD patterns (JCPDS card no. 19-0629) (Fig. 5). The wide-angle XRD peaks of VN-Fe₃O₄-CuO nanocatalyst were easily identified as those of pure cubic phase. Since the impurity peaks were absent, VN-

Fe₃O₄-CuO nanocatalyst of high purity was prepared successfully.

3.2. Catalytic activity and characterization of pyrazolo[3,4-c]pyrazole derivatives

The synthetic route of pyrazolo[3,4-c]pyrazole derivatives is shown in Scheme 2, and the possible mechanism is shown in Scheme 3. The catalyst optimization processes for the synthesis of **1a** are shown in Table 1. The reaction was carried out for 1 min with 10 mol of catalysts, and no yield was obtained. However, when the VN-Fe₃O₄-CuO catalyst was used for 1 min with 20 mol of the catalyst a 10% yield, and 30 mol of catalyst gave a 15% yield. CuO, Cu(OAc)₂, Fe₂O₃, and Fe₃O₄ showed no significant yields at 10 min with 10–30 mol, whereas VN-Fe₃O₄-CuO showed 37, 48, and 82% yields corresponding to 10, 20, and 30 mol in the reaction, respectively. At a reaction time of 20 min, the catalytic performance was not significant even at all concentrations, whereas the VN-Fe₃O₄-CuO catalyst gave yields of 63, 81, and 94% in the presence of 10, 20, and 30 mol of catalyst, respectively. The CuO catalyst in 30 min with 30 mol showed 23% yield; the catalyst Cu(OAc)₂ in 30 min with 30 mol showed 26% yield only; the catalyst Fe₂O₃ in 30 min with 30 mol showed only 32% yield. The highest yield was obtained when



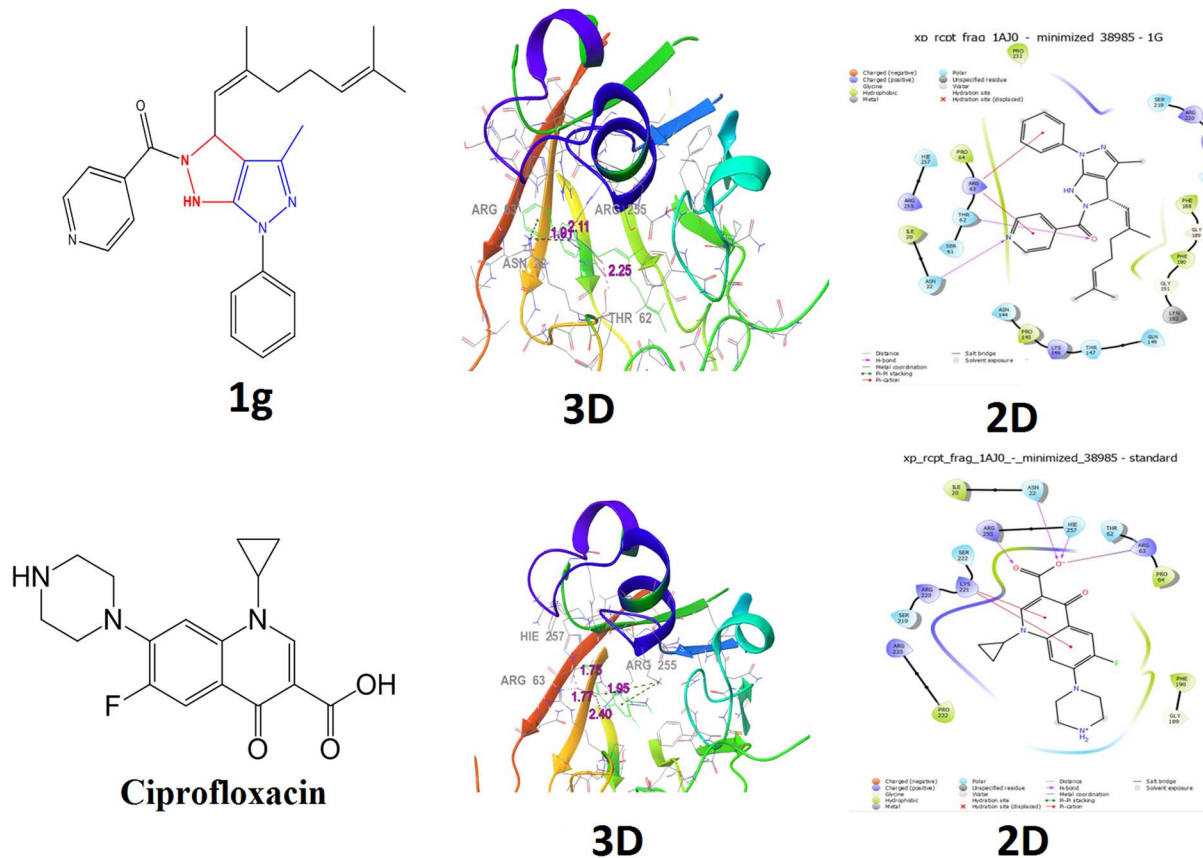


Fig. 8 Molecular docking studies for protein 1AJ0 (*Escherichia coli*) docked with compound **1g** and ciprofloxacin with their 2D and 3D docking structures.

the VN-Fe₃O₄-CuO catalyst was used with 30 mol, at 30 min it gave a 98% yield. The final product of compound **1a** was confirmed by spectroscopic characterization. The IR absorption bands showed that compound **1a** frequency ranged between 3323, 2974, 1655, and 1640 cm⁻¹, corresponding to the -NH, -C=O, and -C=N groups, respectively. For compound **1a**, ¹H NMR spectral value showed a sharp singlet at δ 1.97 for CH₃ proton, δ 8.36 (d, J = 6.23 Hz), 7.83 (d, J = 6.22 Hz), 7.62–7.45 (t, J = 6.21 Hz), 7.30–7.17, 6.13, and 4.0 ppm corresponding to the -Ar ring, -CH, and -NH. ¹³C NMR spectra of compound peaks at δ 172.0, 149.0–105.7, 149.7–121.7, 142.8–123.9, 68.1, and 12.7 ppm corresponding to C=O, pyridine, Ar ring, -CH, and -CH₃ carbons, respectively. The ¹H and ¹³C NMR spectra of (**1a**–**1l**) are included in the ESI file (Fig. S1–S24[†]).

Table 2 shows the results of testing of all the compounds against various fungal species for antifungal activity. Compound **1b** showed high activity against *Candida albicans* (MIC: 0.25 μ g mL⁻¹) than clotrimazole (MIC: 0.5 μ g mL⁻¹). Compound **1h** was effective against *Microsporium audouinii* (MIC: 0.5 μ g mL⁻¹), whereas **1i** (MIC: 2 μ g mL⁻¹) was more active than *Cryptococcus neoformans* with a standard clotrimazole (MIC: 4 μ g mL⁻¹). DMSO was inactive in all fungal and bacterial species.

3.3. Cytotoxic activity

The cytotoxic activity of the synthesized compounds (**1a**–**1l**) was evaluated against the MCF-7 cell line and normal Vero cell line, and all of them showed low activity compared to other standard

Table 4 Molecular docking results for compounds **1b** and **1g**

Compound name	Dock score	Interacting residues	Bond length
Protein 1AI9 (<i>Candida albicans</i>) binds with ligand			
1b	−4.8	—	—
Clotrimazole	−4.4	Phe36	5.38
Protein 1AJ0 (<i>Escherichia coli</i>) binds with ligand			
1g	−3.7	Asn22, Thr62, Arg63, Arg255	1.91, 2.25, 3.24, 2.11
Ciprofloxacin	−5.6	Arg63, Arg255(2), Ile257	1.77, 1.95, 2.40, 1.75



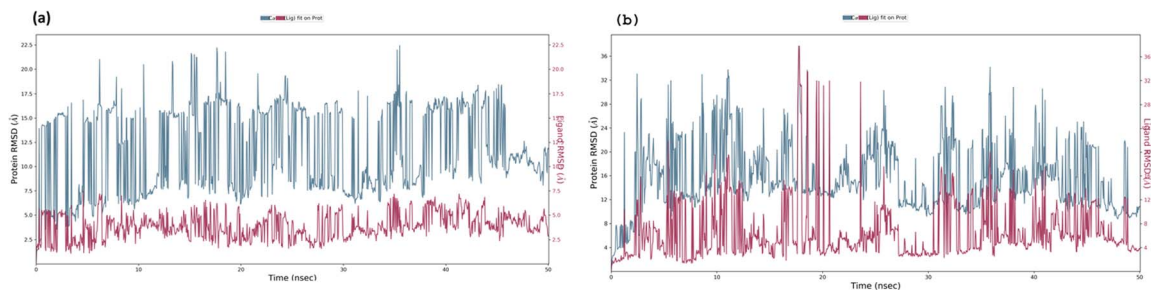


Fig. 9 (a) RMSD plot of 1AI9 docked with **1b**. (b) RMSD plot of 1AJ0 docked with **1g**.

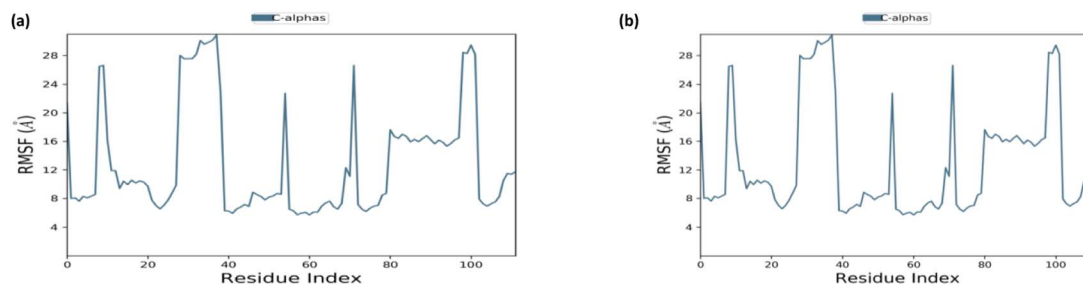


Fig. 10 (a) RMSF plot of 1AI9 docked with **1b**. (b) RMSF plot of 1AJ0 docked with **1g**.

doxorubicin (LC_{50} : $21.05 \pm 0.82 \mu\text{g mL}^{-1}$), as shown in Table 3 (see ESI† for full details). The chosen Vero cell line was based on previously reported literature.^{40,41}

3.4. Docking results

In silico antibacterial and antifungal activity results of the selected ligands against dihydrofolate reductase (DHFR enzyme) from *Candida albicans* (PDB ID: 1AI9)⁴² and *Escherichia coli* were inhibited by docking the synthesized compounds; protein (PDB ID: 1AJ0) was evaluated using the AutoDock Vina program. Dihydrofolate reductase (PDB ID: 1AI9)⁴³ are reported in terms of binding energy and ligand interactions in order to predict the binding energy of ligands within the binding site of the target proteins, as shown in (Fig. 7 and 8).

The docking score of compound **1b** ($-4.8 \text{ kcal mol}^{-1}$) and no bond length compared with those of standard clotrimazole

($-4.4 \text{ kcal mol}^{-1}$) docking score and bond length (5.38). Compound **1b** showed no interacting residues and clotrimazole, as shown in (Fig. 7). The *Candida albicans* binding protein (PDB ID: 1AI9) and the positive control clotrimazole are shown in (Fig. 7). The hydrophobic interactions: GLY 23, LYS 24, MET 25, ILE 33, PHE 36, LYS 57, THR 58, SER 61, ILE 62, LEU 69, ILE 112, GLY 113, GLY 114, ALA 115, and TYR 118. According to the results, clotrimazole, the control, and compound **1b** exhibited equivalent levels of inhibition, as shown in Table 4. The positive control clotrimazole was connected to the *Candida albicans* binding protein (PDB ID: 1AI9) and amino acids ILE 19, MET 25, GLU 32, ILE 33, PHE 36, TH 58, SER 61, ILEV62, LEU 69, ILE 112, GLY 113, GLY 114, and ALA 115, as shown in (Fig. 7). The results showed that compound **1b** possessed inhibition abilities comparable with those of the control ciprofloxacin Table 4.

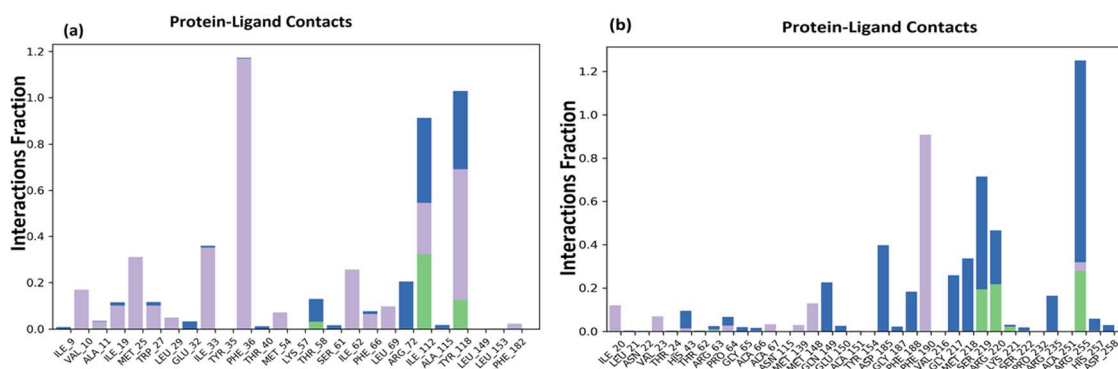


Fig. 11 (a) Histogram of protein–ligand contacts of 1AI9 docked with **1b**. (b) Histogram of protein–ligand contacts of 1AJ0 docked with **1g**.

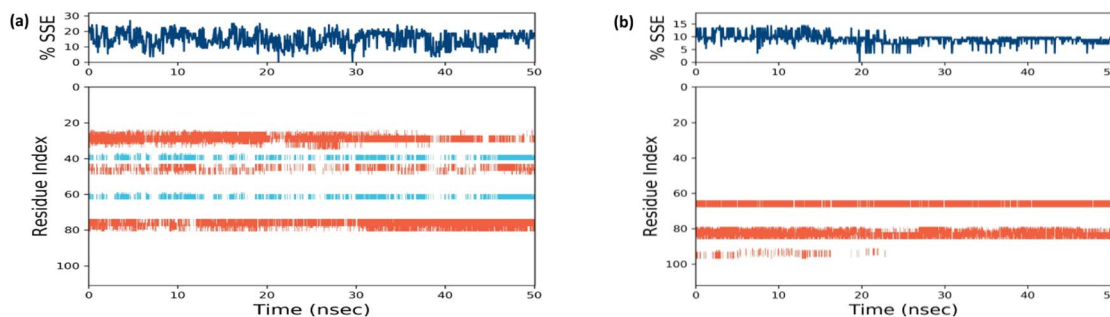


Fig. 12 (a) Timeline representation of protein–ligand contacts of 1A19 docked with **1b**. (b) Timeline representation of protein–ligand contacts of 1AJ0 docked with **1g**.

Compound **1g** had a lower docking score ($-3.7 \text{ kcal mol}^{-1}$), and its bond length is (3.24, 2.25, 2.11, and 1.91), interacting residues (ASN 22, THR 62, ARG 63 and ARG 255). The compound **1g** was connected to the crystal structure of a ternary complex of the *Escherichia coli* binding protein (PDB ID: 1AJ0) and hydrophobic interactions ILE 20, ASN 22, SER 61, THR 62, ARG 63, PRO 64, ASN 144, PRO 145, LYS 146, THRR 147, PHE 188, GLY 189, PHE 190, GLY 191, LYN 192, SER 219, ARG 220, LYS 221, SER 222, ARG 255, and HIE 257 (Fig. 8). Compound **1g** possessed inhibition abilities comparable to those of the control ciprofloxacin with docking score ($-5.6 \text{ kcal mol}^{-1}$), bond length (2.40, 1.95, 1.77, and 1.75), and interacting residues (ARG 63, ARG 255(2), and ILE 257) shown in Table 4.

The standard ciprofloxacin was connected to the crystal structure of a ternary complex of the *Escherichia coli* binding protein (PDB ID: 1AJ0) and hydrophobic interactions ILE 20, ASN 22, THR 62, ARG 63, PRO 64, GLY 189, PHE 190, SER 219, ARG 220, LYS 221, SER 222, PRO 232, ARG 235, and HIE 257, as shown in (Fig. 8).

The control clotrimazole was involved in hydrophobic interactions with (PHE 36) and **1g** (ASN 22, THR 62, ARG 63, and ARG 255) of ciprofloxacin (ARG63, THR62, ARG 225(2), and ILE 257), which are the active site residues and they have multiple functions, including catalysis and substrate binding, as well as stabilising the intermediates and the structure of the binding cleft.

3.5. Molecular dynamics simulation

Docked complex structures of ligands **1b** and **1g** with proteins 1A19 and 1AJ0 were studied using the molecular dynamics simulation by Desmond, Schrödinger to analyze the simulation and stability of the complexes, as shown in Fig. 9–12 (see ESI† for full details).

4. Conclusion

The synthesized $VN\text{-Fe}_3\text{O}_4\text{-CuO}$ nanocatalyst showed high catalytic activity, and the $VN\text{-Fe}_3\text{O}_4\text{-CuO}$ nanocatalyst was used to synthesize pyrazolo[3,4-*c*]pyrazole derivatives. The $VN\text{-Fe}_3\text{O}_4\text{-CuO}$ nanocatalyst was investigated using FTIR, UV, TEM, and XRD. Compound **1g** (MIC: $1 \mu\text{g mL}^{-1}$) was highly active against *Escherichia coli* compared with ciprofloxacin. For antimicrobial

activity, compound **1b** (MIC: $0.25 \mu\text{g mL}^{-1}$) demonstrated a higher antifungal activity against *Candida albicans* than clotrimazole, while compound **1g** (MIC: $1 \mu\text{g mL}^{-1}$) showed a more powerful effect against *Escherichia coli* than ciprofloxacin. Compounds (**1a–1l**) showed significantly low cytotoxicity compared to the standard doxorubicin (LC_{50} : $21.05 \pm 0.82 \mu\text{g mL}^{-1}$). AutoDock Vina was used to consider compounds **1g** and **1b** along with the standards ciprofloxacin and clotrimazole with 1AJ0 and 1A19 proteins. When compared with clotrimazole ($-4.4 \text{ kcal mol}^{-1}$), the molecule **1b** showed a docking score ($-4.8 \text{ kcal mol}^{-1}$), and the novel molecule **1g** displayed a respectable docking score ($-3.7 \text{ kcal mol}^{-1}$) compared with the ciprofloxacin docking score ($-5.6 \text{ kcal mol}^{-1}$) in the 1AJ0 protein. Furthermore, *in silico* analysis indicated that compounds **1b** and **1g** exhibited high docking scores. The results showed that test compounds had outstanding inhibitory abilities in antibacterial and antifungal properties compared to the standards. Therefore, these new pyrazolo[3,4-*c*]pyrazole derivatives could potentially be utilised as lead compounds in the development of novel and clinically useful classes of anti-microbial agents.

Author contributions

J. M.; methodology, A. A.; molecular docking studies, H. M. A.; software analysis, I. A.; investigation and compound characterization. The manuscript was read and approved by all authors prior to publication.

Conflicts of interest

There are no conflicts to declare.

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