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Original Research Article

Synthesis, antimicrobial evaluation and docking studies of new pyrazolone derivatives

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Abstract

Purpose: *To synthesize new antimicrobial azo-pyrazolone derivatives III & IV and evaluate their antimicrobial activities using a combination of in vitro and molecular docking studies.*
Methods: *Azopyrazolone compounds were prepared from the reaction of substituted aniline diazonium with ethyl acetoacetate to give azoxobutyric acid derivatives (II) which were then reacted with phenyl hydrazine or hydrazine hydrate. The pyrazolone derivatives (IV) were acetylated with glacial acetic acid to yield new acetylated pyrazolones (V). An agar dilution method was used to demonstrate the antimicrobial activities of the pyrazolone derivatives and their minimum inhibitory concentration (MIC) values calculated. Molecular docking studies were employed to further evaluate the most active compounds (on the basis of the MICs obtained).*
Results: *The new pyrazolone derivatives showed varying antimicrobial activities (from negligible to strong) against a number of microorganisms. Derivatives IIIb and Vb showed potent activities against Bacillus subtilis, Sarcina lutea, Staphylococcus aureus and Enterococcus faecalis. However, the new compounds did not show antifungal activity. Molecular docking results for compounds IIIb and Vb were consistent with their antimicrobial activities and proved that the compounds inhibited glucosamine-6-phosphate synthase.*
Conclusion: *The new dichloropyrazolone compounds IIIa and Vb possess potent antimicrobial activities. These compounds have promising potential for use as new antibacterial agents or as templates for the design of new antimicrobial drugs.*

Keywords: *Azo-pyrazolone, Dichloropyrazolone, Antimicrobial, Molecular docking*

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INTRODUCTION

Literature survey has revealed that pyrazolone compounds exhibit a variety of biological activities [1-5]. For example, the azo-pyrazolone derivatives have potent cytotoxic [6,7], anti-inflammatory [8-10] and antibacterial activities [11]. Interestingly, pyrazolones **1** and **2** have antimicrobial activities and are considered novel types of non-nucleoside reverse transcriptase inhibitors against HIV-1 [12]. However, drazoxolon **3** is an azo compound containing

isoxazolone ring (ring isostere of pyrazoles) attached to phenyl hydrazone, and is used as a commercial fungicidal agent [13]. In the review of reported biological activities of these derivatives, it was seen that a number of substituted phenylhydrazones bearing a pyrazole ring (**IIIa-c**, **IVa-c** and **Va-c)** have been synthesized (Figure 1).

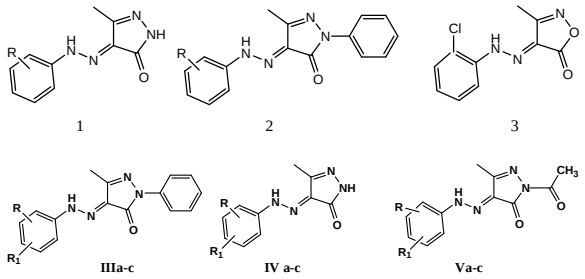

Figure 1: Structures of (a) pyrazolones compounds 1 – 3 and (b) new compounds containing pyrazolones (IIIa-c, IV a-c and Va-c)

The synthesis of new pyrazolone compounds may be a valuable way for producing novel antimicrobial drugs.

EXPERIMENTAL

Instrumentation

Melting points, NMR, IR, mass spectroscopy and elemental composition were measured in accordance with standard procedures reported in the literature [6-9]. All reagents used in this study were purchased from Aldrich Chemical Company (Milwaukee, WI). The authors synthesized compounds II b and IIc, and compounds IVb and IIc in line with reported methods [14-16].

Synthesis of 2-[(3, 4-dimethoxy-phenyl)-hydrazono]-3-oxo-butyric acid ethyl ester (II a)

To a mixture 3,4-dimethoxyaniline (0.01 mol) in aqueous HCl (10 %, 10 mL), an equal volume of ice-cooled $NaNO_2$ solution was added in aliquots for over 20 min with vigorous stirring to afford the diazonium salt. To an ice-cooled solution of the ethyl acetoacetate (0.01 mol) and sodium acetate (0.02mol) in aqueous ethanol (50%, 20 mL), the diazonium salt was added for 3 h with stirring. Following filtration, the product was rinsed in water and purified by crystallization from methanol to yield compound **IIa.**

Method used for synthesizing IIIa-c

A mixture of **IIa-c** (0.015 mol) in absolute ethanol (20 mL), and phenyl hydrazine (0.03 mol) was continually refluxed under heat for 8 h. On cooling, the product was rinsed in water, and after drying, it was subjected to crystallization from acetic acid.

Synthesis of 4-[(3,4-dimethoxy-phenyl)-hydrazono]-5-methyl-2,4-dihydropyrazo-3-one (IV a)

To a solution of **II a** (0.03) in absolute ethanol (20 mL), hydrazine monohydrate (0.03 mol) was added and heated under reflux for 6 h. The reaction was cooled and poured into crushed ice. The precipitate was filtered, washed with water and crystallized from ethanol.

General procedure for synthesis of Va-c

Pyrazolone compounds IV a-c (0.01 mol) were heated under reflux in glacial acetic acid (30 mL) for 8 h. The reaction mixture was cooled to room temperature and added to ice-cooled water (100 mL). The resultant precipitate was filtered, dried and crystallized from ethanol.

Antimicrobial screening

Minimum inhibitory concentration (MIC) was determined using agar dilution according to the procedures outlined by CLSI [17-20].

Molecular modeling studies

Docking experiments were performed using MOE ver. 2010.08. Crystalline structures of glucosamine-6-phosphate (Glcm6P) complexed with glucosamine-6-phosphate synthase (PDB: ID 2VF5) was provided by Protein Data Bank (RCSB). The Docking studies were carried out according to reported methods [6-9].

RESULTS

Spectral data

2-[(3, 4-Dimethoxy-phenyl)-hydrazono]-3-oxo-butyric acid ethyl ester (II a)

Red crystal, yield (85 %); mp 160-162°C; IR (film) 3375 (NH), 2974,2931, 2912 (CH, aliphatic), 1705 (broad band, C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.26-1.32(m, 3H, CH_2CH_3) , 2.37(s, 3H, $COCH_3$), 3.74(s, 3H, 3-OCH_3), 3.79(s,3H.4OCH_3) , 4.20-4.30(m, 2H, -CH_2CH_3) 6.95-7.07 (m, 2H, phenyl H-5,6) 7.13-7.22(m, 1H, phenyl H-2), 11.80 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 14.39, 26.14, 55.88, 60.66, 61.29, 100.73, 107.39, 112.81, 125.14, 135.87, 146.23, 149.97, 163.25, 193.68; analytically calculated values for $C_{14}H_{16}N_4O_4$: C, 55.26; H, 5.30; N,

18.41; obtained values: C, 55.50; H, 5.20; N, 18.50.

4-[(3, 4-Dimethoxy-phenyl)-hydrazono]-5-methyl-2-phenyl-2,4-dihydropyrazol-3-one (III a)

Dark red crystal, yield (60 %); mp 240-242 °C; IR (film) 3425 (NH), 2900 (CH, aliphatic), 1651 (broad band, C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.30 (s, 3H, CH_3), , 3.78(s, 3H, 3-OCH_3), 3.82(s,3H.4OCH_3) , 7.03 (d, J=8.8Hz, 1H, dimethoxyphenyl H-2) 7.14-7.22(m, 2H, dimethoxyphenyl H-5,6), 7.29-7.31(m, 1H, phenyl H-4), 7.43-7.47(m, 2H, phenyl H-3,5), 7.93(d, J= 8Hz, 2H, phenyl H-2,5), 13.38 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.08, 56.10, 56.27, 101.23, 108.87, 112.81, 118.08, 125.14, 127.03, 129.46, 135.44, 138.56, 147.76, 148.70, 150.08, 157.24; analytically calculated values for $C_{18}H_{18}N_4O_3$: C, 63.89; H, 5.36; N, 16.56; obtained values: C, 63.80; H, 5.30; N, 16.60.

4-[(3, 5-Dichloro-phenyl)-hydrazono]-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one (III b)

Pale red crystal, yield (55 %); mp 282-284 °C; IR (film) 3396,3232 (NH), 3089(CH, aromatic), 2900 (CH, aliphatic), 1654 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.30(s, 3H, CH_3), 7.22-7.24(m, 1H, phenyl H-4), 7.38(s, 1H, dichlorophenyl H-4), 7.44-7.46(m, 2H, Phenyl H-3, 5), 7.70- (s, 2H, dichlorophenyl H-2,6), 7.89(d, *J=8 Hz*, 2H, phenyl H-2,6); Mass spectra, $M^{+\cdot}$ = 346, 100%, $[M.+2]^{+\cdot}$ =348, 64%, analytically calculated values for $C_{16}H_{12}$ Cl_2 N_4O: C, 55.35; H, 3.48; N, 16.14; obtained values: C, 55.30; H, 3.20; N, 16.30.

4-[N-(3-methyl--5-oxo-1-phenyl-1,5-dihydropyrazo-4-ylidine)-hydrazino]-benzoic acid ethyl ester (IIIc)

Pale yellow crystal, yield 50 %; mp 250-252 °C; IR (film) 3313 (NH), 3055(CH, aromatic), 2974, 2960 (CH, aliphatic), 1720, 1670 (2 C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.29 (t, *J= 6.8*, 3H, -CH_2CH_3), 2.23 (s, 3H, CH_3) , 4.26(q, *J= 6.8*, 2H, -CH_2CH_3), 7.20-7.23(m, 1H, phenyl H-4), 7.43-7.46(m, 2H, disubstituted-phenyl H-3,5), 7.60-7.63(m, 2H, phenyl H-3, 5), 7.88 (d, 2H, *J= 7.6 Hz*, disubstituted-phenyl H-2, ,H-6), 7.93-7.97(m, 2H, phenyl H-2,6), 13.10 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.15, 14.66, 61.15, 116.40, 118.20, 125.43, 126.61, 129.53, 131.26, 137.20, 138.25,145.25, 147.40, 149.21, 165.60.; analytically calculated values for $C_{19}H_{18}N_4O_3$: C, 65.13; H, 5.18; N, 15.99; obtained values: C, 65.20; H, 5.00; N, 15.70.

Synthesis of 4-[(3, 4-dimethoxy-phenyl)-hydrazono]-5-methyl-2,4-dihydropyrazo-3-one (IVa)

A mixture of IIa (0.03) in absolute ethanol (20 mL) and hydrazine monohydrate (0.03 mol) was refluxed with heating for 6 h. The reaction mixture was cooled and the product was obtained by precipitating in ice. It was thereafter sieved, washed with water and subjected to crystallization in ethanol. Red crystal, yield (80 %); mp 232-234C; IR (film) 3329 (NH), 3020(CH, aromatic), 2966, 2927 (CH aliphatic), 1658 (broad band, C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.15 (s, 3H, CH_3), 3.76 (s, 3H, 3-OCH_3), 3.80 (s,3H.4OCH_3), 6.99 (d, 1H, *J= 8.8 Hz,* phenyl H-5), 7.04 (dd, J_1=*8.8*H_Z, J_2= *2Hz*, 1H, phenyl H-6), 7.19 (s, 1H, *J= 2 Hz,* phenyl H-2), 11.48 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ, 12.05, 56.06, 56.25, 100.80, 108.09, 112.90, 127.44, 135.63, 147.01, 147.22, 150.10, 160.79; analytically calculated values for $C_{12}H_{14}N_4O_3$: C, 54.96; H, 5.38; N, 21.36; obtained values: C, 54.80; H, 5.50; N, 21.40.

2-Acetyl-4-[(3,4-dimethoxy-phenyl)-hydrazono]-5-methyl-2,4-dihydropyrazo-3-one (Va)

Yellowish red crystal, yield (70 %); mp 292-294 °C; IR (film) 3329 (NH), 2962 (CH aliphatic), 1658 (broad band, C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.13 (s, 3H, CH_3), 2.21(s, 3H, $COCH_3$) , 3.77(s, 3H, **3-O**CH_3), 3.81(s,3H.4-OCH_3), 6.99 (d, 1H, *J= 8.8 Hz,* phenyl H-5), 7.06(dd, J_1=8.8H_Z, J_2= 2Hz, 1H, phenyl H-6), 7.20 (d, 1H, *J= 2 Hz,* phenyl H-2), 11.49 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.05, 56.07, 56.26, 100.82, 108.08, 112.92, 127.45, 135.63, 147.01, 147.22, 150.10, 160.79; Anal. Calcd for $C_{14}H_{16}N_4O_4$: C, 55.26; H, 5.30; N, 18.41. Found: C, 55.50; H, 5.20; N, 18.50.

2-Acetyl-4-[(3,5-dichloro-phenyl)-hydrazono]-5-methyl-2,4-dihydro-pyrazol-3-one (Vb)

Reddish yellow crystal, yield (60 %); mp 250-252 °C; IR (film) 3251 (NH), 3062(CH, aromatic), 2900 (CH, aliphatic),1739, 1660(C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.91(s, 3H, CH_3),2.15(s,3H, $COCH_3$), 7.32(s, 1H, phenyl H-4), 7.60 (s, 2H, phenyl H-2,6), 11.61 (s, 1H, NH-N=C).; Mass spectra,, $[M]^{+\cdot}$ = 312, 15.2% $[M+2]^{+\cdot}$ =348, 64%; analytically calculated values for $C_{12}H_{10}$ Cl_2

N_4O_2: C, 46.03; H, 3.22; N, 17.89; values obtained: C, 46.10; H, 3.10; N, 17.80.

4-[N-(1-Acetyl-3-methyl-5-oxo-1,5-dihydropyrazo-4-ylidine)-hydrazino]-benzoic acid ethyl ester (Vc)

Reddish yellow crystal, yield (55 %); mp 296-298 °C; IR (film) 3294 (NH), 2978, 2927 (CH, aliphatic), 1705, 1674 (2 C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.29 (t, *J= 6.8*, 3H, -CH_2CH_3), 1.90 (s, 3H, CH_3), 2.05 (s, 3H, $COCH_3$), 4.24(q, *J= 6.8*, 2H, -CH_2CH_3), 7.46(d, 2H, *J= 8.8,* phenyl H-3,5), 7.89 (d, 2H, phenyl H-2,6), 11.60 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 12.00, 14.60, 21.48, 61.02, 115.59, 125.94, 130.28, 131.18, 131.47, 145.63, 147.35, 160.37, 165.55; analytically calculated values for $C_{15}H_{16}N_4O_4$: C, 56.96; H, 5.10; N, 17.71; values obtained: C, 56.60; H, 5.00; N, 17.70.

Antimicrobial activity of synthesized compounds (MIC)

The compounds showed good antibacterial activities against *Pseudomonas aeruginosa* especially samples IIIb and Vb, which showed high activity against gram positive (*Enterococcus faecalis, Staphylococcus aureus and Sarcina lutea*) and gram negative (*Salmonella typhi*, *E. coli* and *Pseudomonas aeruginosa)* organisms, as well as Gram positive rods (*Bacillus subtilis*). All compounds were inactive against fungi (*Candida albicans*). Their MICs are shown in Table 1.

DISCUSSION

Compound IIa had the characteristic NMR peaks of methyl and ethyl groups. The cyclization of compounds IIa-c with phenyl hydrazine afforded the poly-substituted pyrazolone III. The HNMR of pyrazolones III a-c showed increasing number of aromatic proton peaks and disappearance of the ethyl group peaks. The butyric diazo compound II was reacted with hydrazine hydrate to give the new pyrazolone compounds IV a-c. Disappearance of aliphatic ethyl group HNMR peaks and appearance of NH peak confirmed the structure of compound IV a. The pyrazolone compounds IV a-c were acetylated with glacial acetic acid to produce the new acetylated pyrazolones V a-c. The appearance of new methyl of acetyl group peak and disappearance of NH peak confirmed the structure of compounds Va-c.

The dichlorophenyl group in pyrazolones IIIb and Vb has important role in the antibacterial activities of the pyrazolone compounds. In addition, the substitution at the nitrogen atom of pyrazolone with acetyl or phenyl ring enhances their activities.

To identify the possible mechanism of action of the newly synthesized compounds, they were subjected to molecular docking studies with glucosamine-6-phosphate synthase using the program of MOE site finder. D-glucosamine-6-phosphate (D-Glcm6P) is produced from D-fructose-6-phosphate using L-glutamine as nitrogen source in a reaction catalyzed by glucosamine-6-phosphate synthase. Thus, the inhibition of glucosamine-6-phosphate synthase results in inhibition of production of N-acetylglucosamine which is a major unit in bacterial cell wall building. Consequently, the enzyme is an essential target for antibacterial agents. The docking study was performed using reported methods [6-9]. In this study, the most active compounds **IIIb** and **Vb** from antimicrobial studies were docked into MurA-F binding site to confirm the ability of the novel candidates to act as antibacterial agents.

Table 1: MIC of samples against different microorganisms (µg/mL)

Synthesized compound (200 - 0.7 µg/mL)	Gram -ve rods			Gram +ve rods	Gram +ve cocci			Fungi
	S. typhi	*P. aeruginosa*	*E. coli*	*B. subtilis*	*S. lutea*	*S. aureus*	*E. faecalis*	*C. albicans*
III a	>200	>200	>200	>200	>200	>200	>200	>200
IIIb	>200	>200	>200	≤12.5	≤12.5	≤12.5	≤12.5	>200
IIIc	>200	>200	>200	25	≤12.5	>200	>200	>200
Iva	>200	>200	>200	≤12.5	≤12.5	>200	>200	>200
IVb	>200	≤12.5	>200	>200	>200	>200	>200	>200
IV c	>200	>200	>200	≤12.5	≤12.5	≤12.5	≤12.5	>200
Va	>200	>200	>200	>200	>200	>200	>200	>200
Vb	>200	≤12.5	>200	≤12.5	≤12.5	≤12.5	≤12.5	>200
Vc	>200	>200	>200	≤12.5	≤12.5	>200	≤12.5	>200
Cefotaxime	0.7	6.25	0.7	50	0.7	1.5	>200	>200
Ampicillin	6.25	>200	6.25	6.25	0.7	0.7	1.5	>200
Fluconazole	>200	>200	>200	>200	>200	>200	>200	12.5

Glucosamine-6-phosphate synthetase in complex with Glcm6P as a ligand was sourced from a data bank (PDB: ID 2VF5). The energy (S) of re-docking of Glcm6P into glucosamine-6-phosphate synthase was– 15.39 kcal/mol. Ala602, Val399, Thr302, Gln348, Ser303, Ser349, Ser347 and Thr352 contributed nine H-bonds involving $–NH_2$, –OH and $–PO_4$ groups (Figure 2).

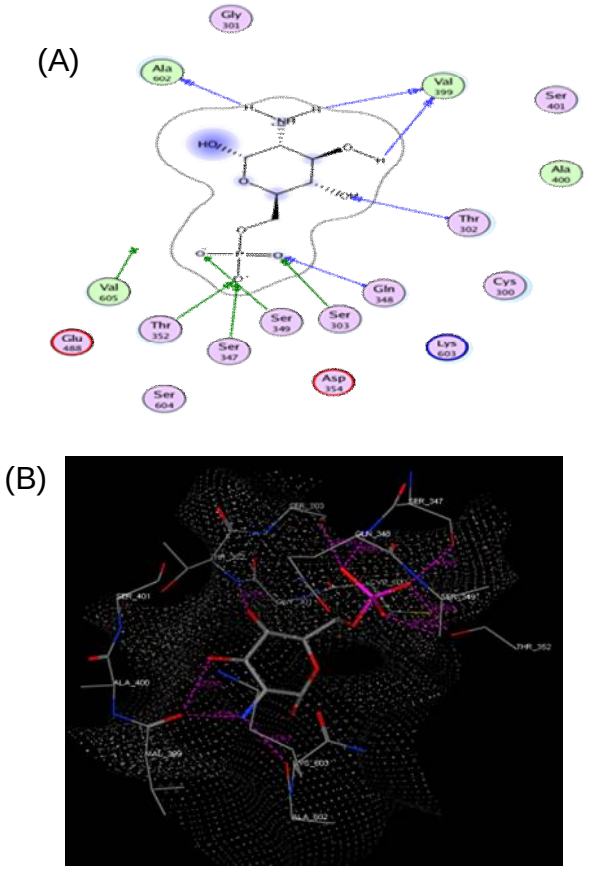

Figure 2: Glcm6P bound inside Glucosamine-6-phosphate synthase. (A) 2D interactions of Glm6P within glucosamine-6-phosphate synthase (H-bonds between $–NH_2$, –OH and $–PO_4$ groups and Ala602, Val399, Thr302, Gln348, Ser303, Ser349, Ser347 and Thr352 amino acids are shown in red line dots. (B) 3D interactions of Glm6P

The results obtained in the docking studies are shown in Table 2. Compound **IIIb** exhibited better docking score (-17.43 kcal/mol) than Glm6P score energy (S) = – 15.39 kcal/mol. Compound **IIIb** showed two hydrogen bonding interactions with Val399 and Ala602 (Figure 3).

Finally, the candidate **Vb** showed an energy score of – 16.33 Kcal/mol and exhibited four hydrogen bonding interactions as follows: (i) Ala602 with NH, (ii) Ser303 with C=O, (iii) Gln348 with C=O and (iv) Thr352 with pyrazole C=O (Figure- 4).

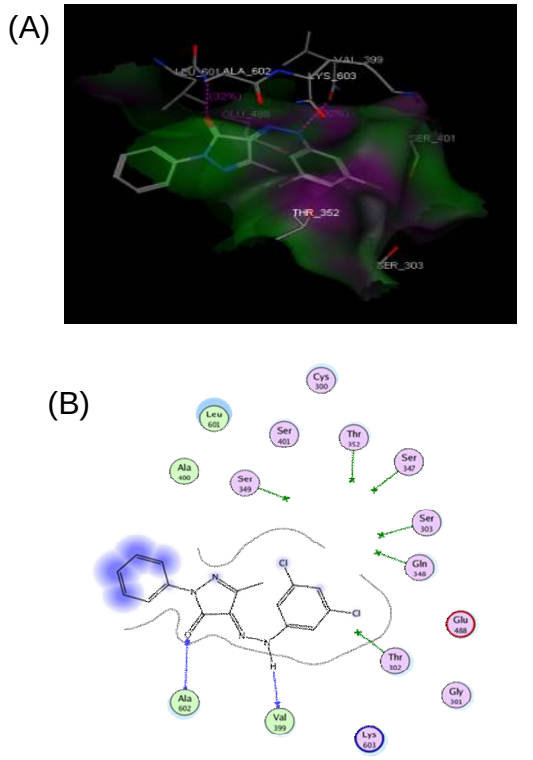

Figure 3: Binding of compound IIIb inside Glucosamine-6-phosphate synthase. (A) 2D interactions of **IIIb** within Glucosamine-6-phosphate synthase, the dotted lines depict two H-bonds between NH with Val399, and C=O with Ala602. (B) 3D interactions of **VIb** with Glucosamine-6-phosphat synthase

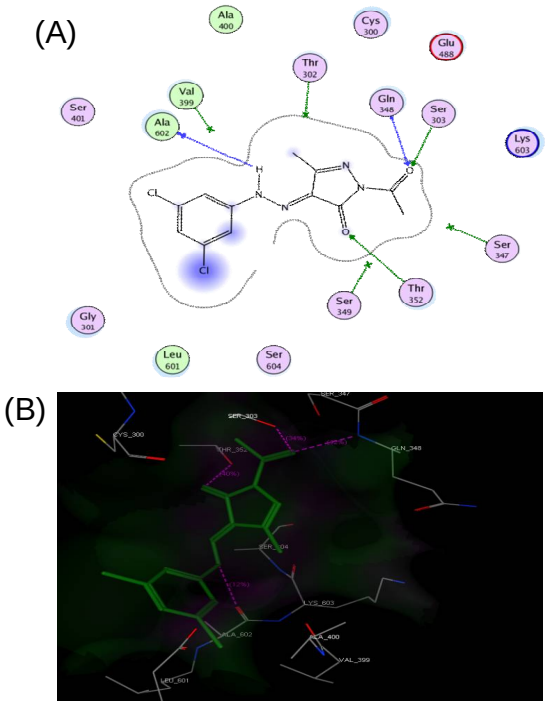

Figure 4: Binding of compound Vb inside glucosamine-6-phosphate synthase. (A) 2D interactions of Vb within Glucosamine-6-phosphate synthase (H-bonds are shown in red dots). (B) 3D interactions of VIII with Glm6P

Table 3: Docking results for IIIb, Vb and Glm6P

Synthesized compound	Affinity (kcal/mol)	H-bonds (n)	Space (Å) from main residue		Group present
IIIb	-17.43	2	Val399	3.24	NH
			Ala602	2.11	C=O
Vb	-16.33	4	Ala602	2.18	NH
			Ser303	3.15	C=O
			Gln348	2.58	C=O
			Thr352	2.48	Pyrazole C=O
Ligand (Glucosamine - 6-phosphate)	-15.39	9	Ala602	2.75	NH
			Val399	2.46, 2.52	NH, OH
			Thr302,	3.41	OH
			Gln348	2.11	P=O
			Ser303	3.15	P=O
			Ser349	3.20	P-O
			Ser347	2.22	P-O
			Thr352	3.21	P-O

CONCLUSION

The new pyrazolones, IIIa-c, IV a-c and V a-c, containing *meta* dichloro phenyl group, have promising antibacterial activity. The relationship between the docking results and their antibacterial activities provides important evidence for the mechanism of the new azopyrazolones as potent inhibitors of glucosamine-6-phosphate synthase. However, further studies are required to screen them for cytotoxic effects.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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