HETEROCYCLES, Vol. 106, No. 12, 2023, pp. -. © 2023 The Japan Institute of Heterocyclic Chemistry
Received, 14th June, 2023 Accepted, 25th October, 2023, Published online, 2nd November, 2023
DOI: 10.3987/COM-23-14877

SYNTHESIS, CHARACTERIZATION, MOLECULAR DOCKING STUDIES AND ANTIMICROBIAL ACTIVITY OF AZOMETHINE DERIVATIVES OF 1,3-SUBSTITUTED PYRAZOLE

Hissana Ather,[a]* Aysha A. Ali Theban,[a] Fareeaa Ashar,[b] Boshra Yahya A. Mohammad Essa,[a] Yomna Ali A. Albarqi,[a] Ghadah Saad A. Alshehri,[a] and Renad Abdulhadi M. Almodawi[a]

[a]Department of Pharmaceutical Chemistry, College of Pharmacy, King Khalid University (KKU), Abha – 62529, Saudi Arabia, Email: hissana@kku.edu.sa
[b]Department of Pharmacy, Annamalai University, Annamalainagar- 608002, TamilNadu, India

Abstract – Two different series of azomethine derivatives of 1,3-substitued pyrazoles were synthesized from the intermediates 3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (**3a**) and 3-(furan-2-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (**3b**) by the condensation of various substituted anilines. The intermediates were obtained from the appropriate phenylhydrazones via Vilsmeier–Haack reaction. The synthesized compounds were characterized by IR, ^{1}H NMR and MASS spectral studies. Molecular docking studies results revealed that **4a7** and **4a8** showed highest binding affinity against fungal target secreted aspartic proteinase (Sap) 1 (**2QZW**) and compounds **4a8** and **4a5** showed highest binding affinity against bacterial target glucosamine-6-phosphate synthase **(2VF5**). *In vitro* antimicrobial activity of corresponding azomethine derivatives were assessed on Gram-positive and Gram-negative bacteria. Antifungal activity was assessed on *Candida albicans*. The results revealed that six out of fifteen compounds screened have shown good antimicrobial activity. Among them, **4a1**, **4b2**, **4a6** and **4a7** shown good inhibition against *Candida albicans*.

INTRODUCTION

The pyrazole (1*H*-pyrazole; 1,2-diazole) nucleus[1] excelled among the nitrogen containing heterocycles due to its diverse biological activities. Pyrazole scaffold fascinated many researchers in medicinal chemistry and from the past two decades noticeable research work done on its synthesis and biological activities. During the last decade a plethora of research papers published on pyrazole scaffold dealing with physical and chemical properties, different synthetic methods,[2-8] biological activities[9,10] and the structure-activity relationship studies.[11]

Pyrazole and its derivatives are one of the most active agents, which exhibit broad spectrum of pharmacological activities such as anti-tubercular,[12] anticonvulsant,[13,14] anti-inflammatory[15,16] and anticancer.[17,18] Recently, it was reported that pyrazole scaffold prodrugs possess bile acid transporting property[19] and nitric oxide donating property.[20] A handful of recent reports demonstrated the antimicrobial activity of pyrazole based synthetic derivatives such as pyrano-pyrazole hybrids,[21] pyrazole-benzimidazole hybrids,[22] pyrazole-hydrazone[23] and pyrazole-aniline[23] derivatives. Pyrazole moiety is present in a large number of natural products having diverse biological activities. So far, the pyrazoline nucleus containing natural products are withasomnine,[24] pyrazofurin, pyrazofurin B, formycin, formycin B, nostocine A, fluviol A, and fluviol B.[25] Besides the presence of pyrazole nucleus in natural products, it is also present as a basic nucleus in the important non-steroidal anti-inflammatory drugs mainly celecoxib, deracoxib and in anti-HIV drug atorividine.[26]

Molecular docking is an essential tool in the Computer-Aided Drug Design. This technique is a part of the so-called "structure-based drug design" (SBDD) methodologies and it was originally developed to anticipate the binding mode of known active compounds and virtually screen vast digital compound libraries in order to minimize the costs and hasten the process of drug development.[27] Molecular docking is a technique that examines how molecules are positioned within the binding site of a macromolecular target. In order to find and generate new, promising molecules, it has been extremely helpful to combine computational and experimental approaches. Modern drug design practices frequently include molecular docking techniques to examine the ligand conformations utilised within the binding sites of macromolecular targets. By analysing crucial aspects of the intermolecular recognition process, this method also calculates the ligand-receptor binding free energy.[28]

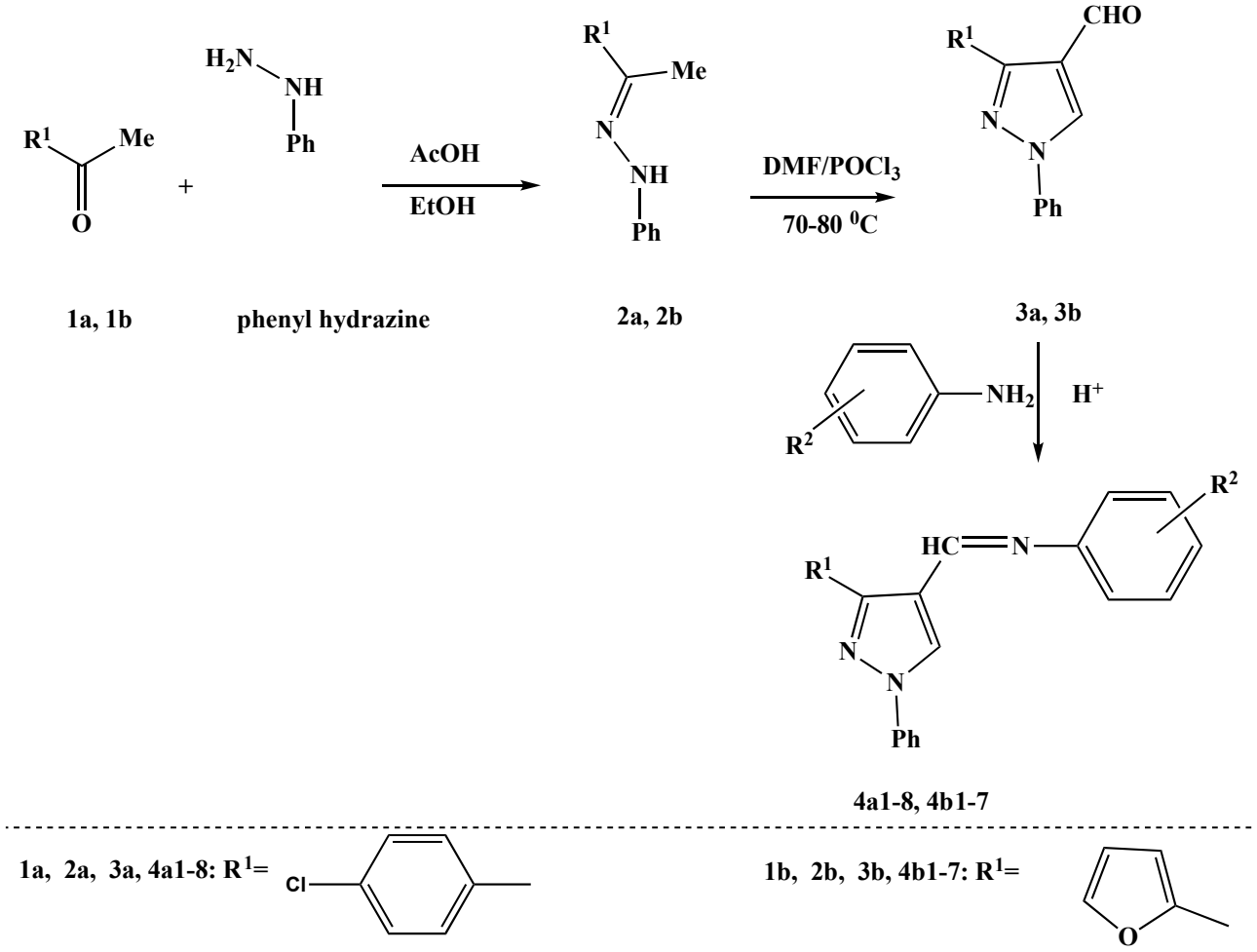
RESULTS AND DISCUSSION

Chemistry

In the present investigation azomethine derivatives of the 1,3-substituted pyrazoles were synthesized in three steps. The first one was the reaction of two different starting materials namely *p*-chloroacetophenone (**1a**) and 2-acetylfuran (**1b**) with phenylhydrazine which results in the formation of two different series of

hydrazones **2a** and **2b** respectively. The hydrazone derivatives **2a** and **2b** treated with Vilsmeier–Haack reagent (DMF–$POCl_3$) leading to the corresponding 4-carbaldehyde functionalized pyrazole intermidiates 3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (**3a)** and 3-(furan-2-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (**3b)** under mild conditions. Three equivalents of the Vilsmeier–Haack reagent (DMF–$POCl_3$) are necessary to obtain the intermediates **3a** and **3b** in good yields.[29]

The two different intermediate aldehydes **3a** and **3b** were functionalized by a classic condensation reaction with various substituted anilines refluxed in ethanol or methanol under mild acidic conditions with trace amounts of concentrated acetic acid to give corresponding azomethine derivatives. The scheme of synthesis of title compounds was enumerated Scheme 1.



4a1, 4b1: R^2 = -H; 4a2, 4b2: R^2 = -4-Me; 4a3, 4b3: R^2 = -4-Cl; 4a4, 4b4: R^2 = -4-Br; 4a5, 4b5: R^2 = -2-Me; 4a6, 4b6: R^2 = -2,6-$(Me)_2$; 4a7, 4b7: R^2 = -3-NO_2, -4-Me; 4a8: R^2 = -3-CF_3

Scheme 1. Synthesis of Azomethine Derivatives of 1,3-Substituted Pyrazoles

Biological evaluation

Fifteen newly synthesized title compounds (**4a1**–**4a8** and **4b1**–**4b7**) were evaluated for their antibacterial activity *in vitro* against Gram-positive bacteria: *S. aureus* and *B. subtilis*; Gram-negative bacteria: *P. aeruginosa*, *E. coli* and antifungal activity against *C. albicans.* The results of preliminary biological screening of all the newly synthesized 1,3- disubstituted pyrazoles against the above strains are presented in Table 1. As we can see, most pyrazoles exhibited moderate antibacterial activity for Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative strains (*P. aeruginosa* and *E. coli*) except **4a2**, **4b5**, **4b6**, **4a8** and **4b1**. Compounds **4a4** and **4a5** did not show any activity to *B. subtilis*. Also, **4a5** showed no potency against *P. aeruginosa*. Interesting results were obtained, all the synthesized 1,3- di substituted pyrazoles revealed moderate to good activity against fungus (*C. albicans*). The minimum inhibitory concentration (MIC) values of the screened compounds are outlined in Table 2. Compound **4a6** with 2,6-dimethylaniline functionalized pyrazole ring exhibited a high potency against *C. albicans* and *E. Coli*, with MIC values of 32 and 64 µg/mL, respectively. This compound is also effective in inhibiting the growth of the rest of microorganism. Compounds **4a7** and **4b2** revealed good antifungal activity against *C. albicans* with MIC value of 32 µg/mL, while compounds **4a5** and **4a4** have a lower degree of potency against all the tested microbes. Compound **4a1** with unsubstituted aniline showed good antifungal activity, and moderate to obvious antibacterial activity.

Table 1. Antimicrobial activity of title compounds

Compound	**Fungi**	**Gram-positive bacterium**		**Gram-negative bacteria**	
	C. albicans	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*
4a1	+++	++	++	++	++
4a2	+	_	_	_	_
4a3	+	++	_	_	_
4a4	+++	_	++	++	++
4a5	+	_	++	_	++
4a6	+++	++	++	++	++
4a7	+++	++	++	++	++
4a8	+	_	_	_	_
4b1	+	_	_	_	_
4b2	+++	++	++	++	++
4b3	+++	++	+	++	++

4b4	++	+	++	+	++
4b5	+	_	_	_	_
4b6	+	_	_	_	_
4b7	++	++	_	++	++

Values are the average of duplicate experiments

Activity was determined from plate-hole diffusion assays with values to indicate the diameters of zones of growth inhibition around the holes. The diameter of zones of inhibition, +++≥ 10 mm, ++ 7-10 mm, + 5-7 mm and - ≤ 5mm.

Table 2. MIC values of antimicrobial activity of the screened compounds

Compound	**MIC (µg/mL)**				
	Fungi	**Gram-positive bacteria**		**Gram-negative bacteria**	
	C. albicans	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*
4a1	32	64	128	128	64
4a4	128	128	64	128	128
4a5	128	128	64	64	128
4a6	32	128	128	128	64
4a7	32	128	128	128	128
4b2	32	128	128	128	128
Ciprofloxacin	2.0	0.25	0.125	2.0	16

Minimum inhibitory concentration of the screened compounds determined using broth tube technique, values were the average ones of duplicate experiments. Ciprofloxacin was used as a control.

Molecular docking studies

All the synthesized compounds were docked into the active site of glucosamine-6-phosphate synthase (**2VF5**) and secreted aspartic proteinase (Sap) 1 (**2QZW**) to determine the binding affinity of the compounds. Docking scores of synthesized compounds for glucosamine-6-phosphate synthase (2VF5) were ranging from -7.6 to-8.4 kcal/mol as contrast to docking score of standard glucosamine-6-phosphate -4.5 kcal/mol; whereas, for secreted aspartic proteinase (Sap) 1 (2QZW) it is ranging from -6.8 to -7.8 as contrast to Naftifine reference drug binding score -6.7 kcal/mol. The binding scores of the compounds are mentioned in Tables 3 and 4.

Table 3. Molecular docking scores of synthesized compounds against glucosamine-6-phosphate synthase (PDB ID:2VF5)

S. No	Compound	binding affinity (-kcal/mol)	Hydrophobic interactions and Hydrogen bond interactions
1	**4a1**	-8.0	Asp-474, Glu-569, Leu-525, Arg-472, Ala-572, Ala-520
2	**4a2**	-7.6	Leu-484, Ile-326, Leu-601, Val-605, Cys-300, Asp-354, Ala-353
3	**4a3**	-7.7	Leu-601, Cys-300, Gly-301, Lys-487, Leu -484, Leu-480
4	**4a4**	-7.6	Arg-472, Asp-474, Ala-520, Ala-551, Tyr-576, Ala-572, Val-567
5	**4a5**	-8.1	Arg-472, Asp-474, Ala-520, Ala-572, Glu-569, Leu-525
6	**4a6**	-7.7	Ala-551, Asp-474, Ala-520, Tyr-576, Val-567, Ala-572
7	**4a7**	-7.7	Arg-472, Asp-474, Ala-572, Ala-520, Tyr-576, Val-567, Ala-551
8	**4a8**	-8.4	Pro-521, Ala-520, Glu-569, Arg-472, Ala-572, Leu-525, Asp-548
9	**4b1**	-7.6	Val-567, Ala-572, Ala-551, Asp-548
10	**4b2**	-7.8	Arg-472, Ala-520, Val-567, Asp-474, Ala-572, Tyr-576,
11	**4b3**	-7.8	Tyr-312, Leu-525, Arg-472, Val-567, Ala-572, Ala-520
12	**4b4**	-7.7	Arg-472, Ala-572, Ala-520, Tyr-312
13	**4b5**	-7.7	Ala-551, Asp-548, Ala-572, Val-567, Pro-521, Ala-520
14	**4b6**	-7.7	Ala-520, Ala-551, Asp-548, Ala-572, Val-567
15	**4b7**	-7.9	Leu-525, Arg-472, Ala-572, Ala-520, Tyr-312
Standard	Glucosamine-6-phosphate	-4.5	Glu-396, Ser-604, Gln-348

Table 4. Molecular docking scores of synthesized compounds against secreted aspartic proteinase (Sap) 1 (PDB ID:2QZW)

S. No	Compound	binding affinity (-kcal/mol)	Hydrophobic interactions/ Hydrogen bond interactions
1	**4a1**	-7.1	Phe-251, Leu-283, Val-253, Lys-243, Tyr-291, Tyr-285, Ala-286
2	**4a2**	-7.5	Phe-251, Leu-283, Lys-243, Asp-245
3	**4a3**	-7.1	Ile-123, Ile-30, Asp-218, Ala-303, Ile-305, Leu-216, Ile-82, Ala-133
4	**4a4**	-7.0	Ile-82, Ala-133, Ile-123, Ile-30, Asp-218, Ala-303, Ile-305, Leu-216
5	**4a5**	-6.9	Ala-286, Tyr-285, Phe-251, Tyr-291, Lys-243, Val-253
6	**4a6**	-7.2	Tyr-285, Phe-251, Tyr-291, Lys-243, Val-253, Leu-283
7	**4a7**	-7.8	Val-12, Pro-120, Ile-123, Arg-51
8	**4a8**	-7.6	Ile-82, Ile-123, Ile-119, Ile-30, Ile-305, Tyr-225, Thr-221
9	**4b1**	-7.1	Pro-162, Arg-312, Ala-281, Leu-297
10	**4b2**	-7.4	Leu-297, Ala-281, Arg-312, Pro-162
11	**4b3**	-6.8	Ile-82, Ala-133, Ile-123, Ile-30, Asp-218, Ala-303, Ile-305, Leu-216, Ala-133
12	**4b4**	-7.3	Leu-297, Ala-281, Ala-281, Pro-162
13	**4b5**	-6.8	Lys-243, Phe-251, Leu-283, Ala-286, Ser-284
14	**4b6**	-6.9	Ser-244, Gly-246, Leu-283, Phe-251, Lys-243, Tyr-291, Ser-284, Ala-286
15	**4b7**	-7.3	Lys-108, Tyr-137, Val-110, Leu-76, Asp-138, Asn-146, Thr-143, Lys-96
Standard	Naftifine	-6.7	Lys-243, Leu-283, Asp-245, Phe-251

Molecular docking simulations of compounds against glucosamine-6-phosphate synthase (PDB ID: 2VF5)

Among 15 compounds, **4a8** displayed highest binding affinity -8.4 kcal/mol, as compared standard drug glucosamine-6-phosphate binding affinity -4.5 kcal/mol for glucosamine-6-phosphate synthase (**2VF5**) the reason could be the formation of two conventional hydrogen bonds with amino acid Asp-548. **4a8** forms a carbon hydrogen bond with Glu-569 amino acid and hydrophobic interaction with Pro-521, Ala-520, Arg-472, Ala-572, Leu-525.

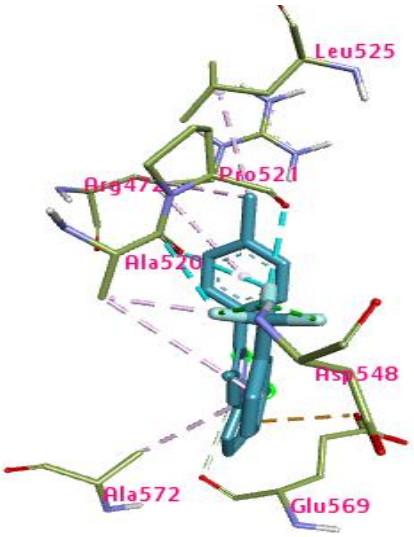


Figure 1. 3D Binding interactions of **4a8**

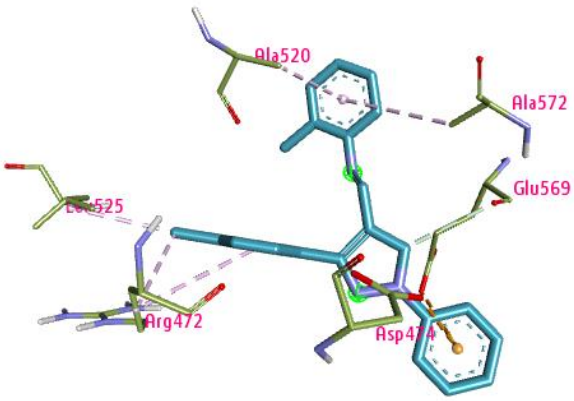


Figure 2. 3D Binding interactions of compound **4a5**

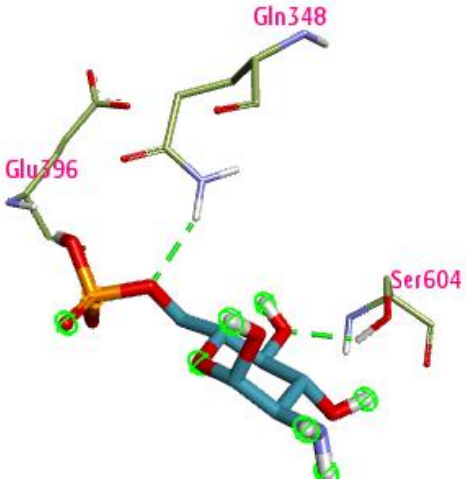


Figure 3. 3D Binding interactions of glucosamine-6-phosphate

Figure 1 to 3 represent 3D binding interactions of compounds against glucosamine-6-phosphate synthase. **4a5** displayed binding affinity -8.1 kcal/mol, as compared to standard drug for glucosamine-6-phosphate synthase (**2VF5**). **4a5** forms one carbon hydrogen bond with Glu-569. Pi-alkyl and alkyl bonds with Arg-472, Ala-520, Ala-572, & Leu-525, one anion-pi bond with amino acid Asp-474. Glucosamine-6-phosphate showed binding affinity -4.5 kcal/mol for glucosamine-6-phosphate synthase (**2VF5**). It forms total of three conventional hydrogen bonds with amino acids Glu-396, Gln 348 and Ser 604.

Molecular docking simulations of compounds against secreted aspartic proteinase (Sap) 1 (PDB ID: 2QZW)

Among 15 compounds, **4a7** demonstrated highest binding affinity -7.8 kcal/mol, as compared reference drug Naftifine binding affinity -6.7 kcal/mol for secreted aspartic proteinase (Sap) 1 (**2QZW**). **4a7** forms one conventional hydrogen bond with amino acid Arg 51. One pi-sigma bond with Ile-123, one cation-pi bond with Arg 51, one alkyl bond with Val 12 and one pi-alkyl bond with Pro 120. Figures 4 to 6 indicates the 3D binding interactions of compounds against secreted aspartic proteinase (Sap) 1.

Compound **4a8** demonstrated binding affinity -7.6 kcal/mol. **4a8** made one pi-donor hydrogen bond with Thr 221, one pi-sigma bond with Ile 123. pi-alkyl bonds made with amino acids Ile 82, Ile 305, Tyr 225, Ile 119, Ile 30.

Naftifine reference standard showed binding affinity -6.7 kcal/mol for secreted aspartic proteinase (Sap) 1 (**2QZW**). It forms three pi-pi T-shaped bond with amino acid Phe 251, one anion-pi bond with Asp 245, and pi-alkyl bonds with Leu 283, & Lys 243.

Docking studies results are correlating with *in vitro* antimicrobial activity as **4a7** and **4a8** exhibited highest binding score against fungal target secreted aspartic proteinase (Sap) 1 (2QZW). Compounds **4a8** and **4a5** demonstrated highest binding score against bacterial target glucosamine-6-phosphate synthase (2VF5).

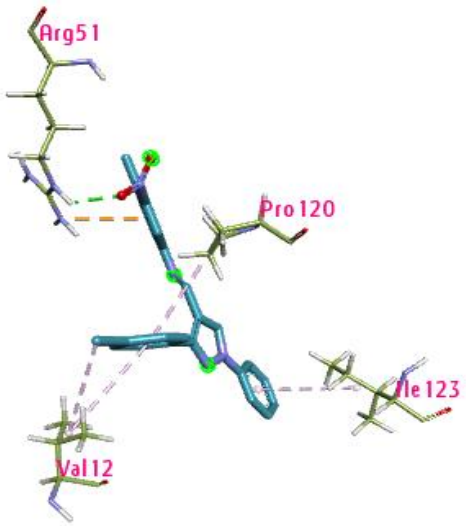


Figure 4. 3D Binding interactions of **4a7**

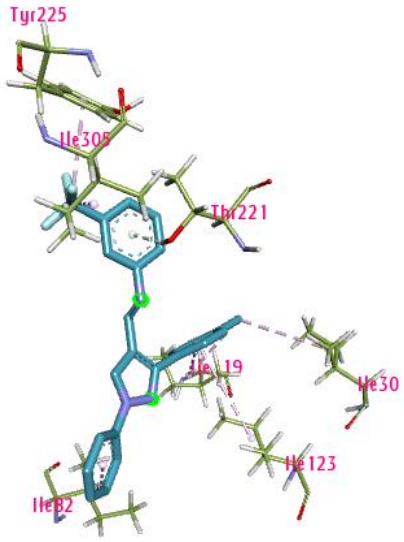


Figure 5. 3D Binding interactions of **4a8**

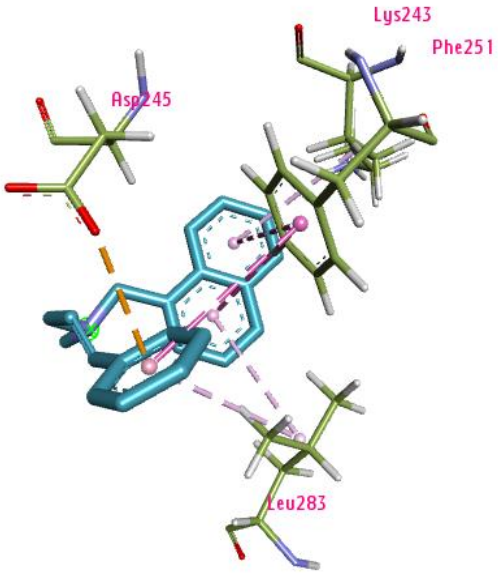


Figure 6. 3D Binding interactions of Naftifine

CONCLUSIONS

In conclusion two different series of azomethine derivatives of 1,3- substituted pyrazoles were synthesized, characterized and evaluated for their antimicrobial activity. In addition, predicted binding affinities of compounds using molecular docking studies. The preliminary antimicrobial screening of the fifteen synthesized compounds revealed that only 6 compounds showed moderate antimicrobial activity. The compounds **4a1**, **4b2**, **4a6** and **4a7** revealed good potency against *C. albicans* with MIC value of 32 µg/mL. In the molecular docking studies all the compounds exhibited good binding affinity towards targets, among

all the compounds **4a7**, **4a8** and **4a5** showed highest binding affinity towards targets, which reveals that these compounds may have potential to become lead antifungal agents upon further investigation focusing on the molecular mechanism of the inhibition of fungal growth and structural activity relationship studies.

EXPERIMENTAL

All the reagents and chemicals used in the current work were purchased from Sigma Aldrich, Sd-Fine chem and Research labs. All the materials were further purified before use. The solvents used were purified by distillation before the usage. Melting points were determined by Remi electronic melting point apparatus. TLC plates were obtained from Merck-precoated aluminum sheets of silica gel 60 F254 of 0.5 mm thickness. For the visualization of TLC spots iodine, UV, anisaldehyde, H_2SO_4 were used. IR spectra were recorded on Agilent FTIR by KBr pellet method. ^{1}H NMR recorded on BRUKER DRX–500 MHz. The splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), q (quartet) m (multiplet). MASS recorded on SHIMADZU MS.

Synthesis

Synthesis of hydrazones 2a and 2b

To a solution of *p*-chloroacetophenone (3.7 g, 24 mmol) in EtOH (90 mL) in a round bottomed flask, glacial acetic acid (1 mL) and phenylhydrazine hydrochloride (3 g, 20 mmol) were added. Then the mixture was refluxed for 1 h. After cooling, the reaction mixture was poured into ice-cold water and the precipitate formed was filtered, dried under vaccum and crystallized from EtOH.[30]

Synthesis of 3a and 3b (Vilsmeier-Haack Reaction)

The solution of **2a** (3 g) or **2b** (3 g) in DMF (3 mL) was added dropwise to the mixture of DMF (2.58 g, 35.3 mmol) and $POCl_3$ (5.4 g, 35.3 mmol) which was previously cooled at 0 ^{o}C. Then the reaction mixture was heated slowly at 70–80 ^{o}C for 5 h. After cooled to room temperature reaction mixture was basified with cool saturated aqueous K_2CO_3 solution. The precipitate washed with water and crystallized from EtOH.[29,31]

General procedure for preparation of 4a and 4b

Compound **3a** (0.5 g) or **3b** (0.5 g) were dissolved in MeOH (15 mL) and 0.5 mL of glacial acetic acid was added to it. Then 2.2 mmol of various substituted anilines were added and the reaction mixture was refluxed for 6 h. After cooling at room temperature, the precipitate was filtered and washed with cold water and crystallized from EtOH.[31]

The physical and spectral data of the synthesized compounds are given below:

Compound-4a1: *N*-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)aniline: Pale yellow solid; mp 161-162 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.4-7.65 (m, 10H, Ar-H), 7.05 (t, 1H, Ar-H),

6.95 (d, 2H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 3125 (C-H str), 1620 (C=C str), 1645 (C=N str), 1725 (C-N str), 699 (C-H bend); MS: *m/z* = 358.8 (M^+) for $C_{22}H_{16}ClN_3$.

Compound-4a2: *N*-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-4-methylaniline: Pale yellow crystalline solid; mp 168-169 °C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 2.35 (s, 3H, CH_3), 7.2-7.25 (m, 4H, Ar-H), 7.45-7.65 (m, 8H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 765-785 (C-Cl str), 3126 (C-H str), 1630 (C=C str), 1645 (C=N str), 1725 (C-N str), 699 (C-H bend); MS: *m/z* = 372.8 (M^+) for $C_{23}H_{18}ClN_3$.

Compound-4a3: 4-Chloro-*N*-((3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)aniline: White crystalline solid; mp 159-160 °C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.45-7.65 (m, 10H, Ar-H), 7.05 (d, 2H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 650-670 (C-Br str), 3126 (C-H str), 1630 (C=C str), 1645 (C=N str), 1725 (C-N str), 699 (C-H bend); MS: *m/z* = 393.1 (M^+) for $C_{22}H_{15}Cl_2N_3$.

Compound-4a4: 4-Bromo-*N*-((3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)aniline: Pale yellow crystalline solid; mp 174-175 °C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 8H, Ar-H), 7.20 (d, 2H, Ar-H), 7.70 (d, 2H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 765-785 (C-Cl str), 3126 (C-H str), 1630 (C=C str), 1645 (C=N str), 1725 (C-N str), 699 (C-H bend); MS: *m/z* = 437.1 (M^+) for $C_{22}H_{15}BrClN_3$.

Compound-4a5: *N*-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-2-methylaniline: Pale yellow crystalline solid; mp 165-166-164 °C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 8H, Ar-H), 6.9 (t, 1H, Ar-H), 7.05 (d, 1H, Ar-H), 7.95 (d, 2H, Ar-H), 7.2-7.3 (m, 2H, Ar-H), 8.4 (s, 1H, pyrazole), 2.35 (s, 3H, CH_3); IR (KBr *v*max cm^{-1}): 765-785 (C-Cl str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 372.3 (M^+) for $C_{23}H_{18}ClN_3$.

Compound-4a6: *N*-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-2,6-dimethylaniline: Pale yellow crystalline solid; mp 179-180 °C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 9H, Ar-H), 7.0 (d, 2H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole), 2.35 (s, 6H, CH_3); IR (KBr *v*max cm^{-1}): 765-785 (C-Cl str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 386.2 (M^+) for $C_{24}H_{20}ClN_3$.

Compound-4a7: *N*-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-4-methyl-3-nitroaniline: Pale yellow crystalline solid; mp 183-184 °C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 10H, Ar-H), 7.8 (s, 1H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole), 2.35(s, 3H, CH_3); IR (KBr *v*max cm^{-1}): 765-785 (C-Cl str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 417.6 (M^+) for $C_{23}H_{17}ClN_4O_2$.

Compound-4a8: *N*-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-3-(trifluoromethyl)aniline: Pale yellow crystalline solid; mp 176-177 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.70 (m, 11H, Ar-H), 6.9 (d, 1H, Ar-H), 7.95 (d, 2H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 1185-1205 (C-F str), 765-785 (C-Cl str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 426.6 (M^+) for $C_{23}H_{15}ClF_3N_3$.

Compound-4b1: *N*-((3-(Furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)aniline: Pale yellow crystalline solid; mp 141-142 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40 – 7.70 (m, 9H, Ar-H), 6.9 - 7.0 (m, 3H, Ar-H), 7.1 (t, 1H, Ar-H), 7.8 (d, 1H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 314.6 (M^+) for $C_{20}H_{15}N_3O$.

Compound-4b2: *N*-((3-(Furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-4-methylaniline: Yellow crystalline solid; mp 148-149 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.70 (m, 7H, Ar-H), 7.2 (d, 2H, Ar-H), 7.3 (d, 2H, Ar-H) 7.8 (d, 1H, Ar-H), 6.95(t, 1H, Ar-H), 8.4 (s, 1H, pyrazole), 2.33 (s, 3H, CH_3); IR (KBr *v*max cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 328.2 (M^+) for $C_{21}H_{17}N_3O$.

Compound-4b3: 4-Chloro-*N*-((3-(furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)aniline: Pale yellow crystalline solid; mp 153-154 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 9H, Ar-H), 6.9-7.05 (m, 3H, Ar-H), 7.8 (d, 1H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 348.1 (M^+) for $C_{20}H_{14}ClN_3O$.

Compound-4b4: 4-Bromo-*N*-((3-(furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)aniline: White solid; mp 166-167 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 7H, Ar-H), 6.9 (t, 1H, Ar-H), 7.2 (d, 2H, Ar-H), 7.75 (d, 2H, Ar-H), 7.85 (d, 1H, Ar-H), 8.4 (s, 1H, pyrazole); IR (KBr *v*max cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 393.05 (M^+) for $C_{20}H_{14}BrN_3O$.

Compound-4b5: *N*-((3-(Furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-2-methylaniline: Pale yellow solid; mp 149-150 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 7H, Ar-H), 6.9 (t, 1H, Ar-H), 7.2-7.25 (m, 2H, Ar-H), 7.05 (d, 1H, Ar-H), 6.8 (t, 1H, Ar-H), 7.85 (d, 1H, Ar-H), 8.4 (s, 1H, pyrazole), 2.3 (s, 3H, CH_3); IR (KBr *v*max cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 328.15 (M^+) for $C_{21}H_{17}N_3O$.

Compound-4b6: *N*-((3-(Furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-2,6-dimethylaniline: White crystalline solid; mp 162-163 ^{o}C; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.45-7.65 (m, 8H, Ar-H), 6.9 (t, 1H, Ar-H), 7.05 (d, 2H, Ar-H), 7.85 (d, 1H, Ar-H), 8.4 (s, 1H, pyrazole), 2.3(s, 6H, CH_3); IR (KBr *v*max

cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 342.15 (M^+) for $C_{22}H_{19}N_3O$.

Compound-4b7: *N*-((3-(Furan-2-yl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-4-methyl-3-nitroaniline: White crystalline solid; mp 171-172 ºC; ^{1}H NMR: (500 MHz, $CDCl_3$, ppm) δ 7.40-7.65 (m, 9H, Ar-H), 6.9 (t, 1H, Ar-H), 7.8 (d, 1H, Ar-H), 7.9 (s, 1H, Ar-H), 8.4 (s, 1H, pyrazole), 2.3(s, 3H, CH_3); IR (KBr *v*max cm^{-1}): 1145-1160 (C-O str), 3120-3140 (C-H str), 1620-1640 (C=C str), 1645-1660 (C=N str), 1720-1735 (C-N str), 699 (C-H bend); MS: *m/z* = 373.05 (M^+) for $C_{21}H_{16}N_4O_3$.

Pharmacology

The following microorganisms were taken for detecting antimicrobial activity: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922.

The preliminary screening for antibacterial as well as antifungal activity of the fifteen tested compounds in DMSO (25 mg/mL) was evaluated using diffusion plate assay.[32,33] A two-step serial dilution technique[34] was adopted to determine the minimum inhibitory concentration (MIC) values of the screened compounds. The MIC value was considered as quantitative method and was used for evaluation of the antimicrobial activity of the synthesized compounds against the given microbes.

Preliminary screening for antimicrobial activity

Suspensions of the above-mentioned microorganisms were prepared by inoculating fresh stock cultures into separate broth tubes, each containing (8 mL) of Mueller–Hinton broth for colonies of bacterial strains and fungal strains. The inoculated tubes were incubated at 37 and 28 ºC for 24 h, for bacterial and fungal strains, respectively. Then, 250 µL of bacterial culture (10^6–10^8 bacteria every mL) was added aseptically to 20 mL of solid culture (a mixture of tryptone, Sabouraud dextrose agar and yeast extract) at 45 ºC, mixed well, and poured into a sterile petri dish (90 mm). A sterile cork-borer of 6 mm diameter was used to make 2 wells on each of the Petri dish. One was filled with 10 µL of the screened compounds. In order to check the effect of the solvent, a control test was also performed containing only DMSO. Then the cultures were incubated at 37 and 28 ºC for 24 h for the bacterial and fungal strains. After incubation, the zone of inhibition on the plates was recorded. The compounds with the diameter of the growth-inhibition zones greater than 5 mm were considered positive and can be subjected for further determination of the minimum inhibitory concentration (MIC) values. The test was repeated three times for each compound. All the new compounds (**4a1-4a8** and **4b1-4b7**) were subjected to preliminary biological screening.

Determination of the minimum inhibitory concentration (MIC)

Broth tube dilution method[34,35] was followed to determine the MIC values for all the compounds subjected to screening against the microorganisms, and for comparison ciprofloxacin was used as a reference. For sample preparation, each of the test compounds and reference were dissolved in DMSO at a concentration

of 1.280 µg/mL, and further dilutions of the compounds and reference in culture medium MHB (Mueller-Hinton Broth) were prepared at the required quantities of 128, 64, etc., down to 0.125 µg/mL. Bacterial strains and fungal strains were maintained on MHA (Mueller-Hinton Agar) medium at 37 and 28 °C for 4 h, respectively. The inocula of microbes were prepared by suspension in 10 mL of culture medium for colonies from culture on MHA. The cell density of each inoculum was adjusted in culture medium of a 0.5 McFarland standard. The final amount applied was 10^5 CFU/mL for bacteria and fungi. The microbial inocula were added to the two-step diluted samples. MIC values were read after incubation at 37 and 28 °C for bacterial and fungal strains, respectively. After 24 h, the last tube with no growth of microorganisms was recorded to represent the MIC value expressed in mg/mL. All experiments were carried out three times.

Molecular docking studies

Protein preparation

AutoDock Vina 4.2 with the standard protocol was used to dock the proteins (PDB ID: **2VF5**; and PDB ID: **2QZW)** and incorporated the synthesized compounds into the active site of proteins. The crystal structure of glucosamine-6-phosphate synthase (PDB: 2VF5) and secreted aspartic proteinase (Sap) 1 (PDB: 2QZW) were retrieved from RCSB PDB bank (https://www.rcsb.org/), and removed water molecules and its endogenous ligand. The protein was prepared using the protein preparation wizard AutoDock 4.2 (MGLTools 1.5.7). The unwanted water molecules were deleted, added polar hydrogens, Kollmann and Giester charges, and also bond orders were assigned; finally converted to PDBQT format. The center grid box X, Y, and Z coordinates (34.34 A˚, 19.42 A˚, and 0.90 A˚), and (-9.36 A˚, 11. 64 A˚, and -17.84 A˚) for 2VF5, and 2QZW were used to carry out docking simulation. Calculated ligands torsion angles to assign the flexible and non-bonded rotation of molecules. All of the compounds in our data collection were docked in the protein's active site under investigation, and the findings were evaluated using Biovia Discovery Studio 4.0.

Ligands selection

Designed total fifteen compounds (**4a1-4a8** and **4b1-4b7**) using ChemDraw software in SDF 3D format. Converted SDF files into PDB format using ChemBio 3D. Added partial charges (Giester and Kollmann) using Auto Dock. Chosen torsion tree and set the torsions to five then saved in PDBQT 3D format.

Molecular docking

The molecular docking investigation was carried out by genetic algorithm using the AutoDock Vina programme. We used Perl script to do virtual screening in Autodock Vina (Command-perl vina_windows.pl). Designed compounds were docked with glucosamine-6-phosphate synthase (PDB: **2VF5**) and secreted aspartic proteinase (Sap) 1 (PDB: **2QZW)**. Afterward, the ligand docked-out files, were inputed into Biovia Discovery Studio 4.0 to visualize for 2D-3D interactions of ligands with the

targets.The docking scores of the compounds were represented kcal/mol, where more negative energy means stronger binding.

ACKNOWLEDGEMENTS

The authors extend their sincere appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through Small Groups (Grant number RGP1/57/44).

FUNDING

This work was supported by Deanship of Scientific Research at King Khalid University [RGP1/57/44].

REFERENCES

1. B. Stanovnik and J. Svete, *Sci. Synth*., 2002, **12**, 15.
2. L. C. Behr, R. Fusco, and C. H. Jarboe, *The Chemistry of Heterocyclic Compounds, John Wiley & Sons: New York, NY, USA*, 1967, 150.
3. A. R. Katritzky and C. W. Rees, *Comprehensive Heterocyclic Chemistry, Pergamon Press, New York*, 1984, **5**, 167.
4. A. R. Katritzky, C. W. Rees, and E. F. V. Scriven, *Comprehensive Heterocyclic Chemistry II, Pergamon Press, New York*, 1996, **3**, 1.
5. K. Makino, H. S. Kim, and Y. Kurasawa, *J. Heterocycl. Chem*., 1999, **36**, 321.
6. K. Makino, H. S. Kim, and Y. Kurasawa, *J. Heterocycl. Chem*., 1998, **35**, 489.
7. K. Takagi and M. Hubert-Habart, *J. Heterocycl. Chem*., 1996, **33**, 1003.
8. M. H. Elnagdi, G. E. H. Elgemeie, and F. A. -E. Abd-Elaal, *Heterocycles*, 1985, **23**, 3121.
9. S. Malladi, A. M. Isloor, S. K. Peethambar, B. M. Ganesh, and P. S. K. Goud, *Der Pharma Chem*., 2012, **4**, 43.
10. H. B. Bhatt and S. Sharma, *Arabian J. Chem*., 2013, **10**, S1590.
11. R. C. Khunt, V. M. Khedkar, R. S. Chawda, N. A. Chauhan, A. R. Parikh, and E. C. Coutinho, *Bioorg. Med. Chem. Lett*., 2012, **22**, 666.
12. R. B. Pathak, P. T. Chovatia, and H. H. Parekh, *Bioorg. Med. Chem. Lett*., 2012, **22**, 5129.
13. M. Abdel-Aziz, G. E. A. Abuo-Rahma, and A. A. Hassan, *Eur. J. Med. Chem*., 2009, **44**, 3480.
14. D. Kaushik, S. A. Khan, G. Chawla, and S. Kumar, *Eur. J. Med. Chem*., 2010, **45**, 3943.
15. G. Daidone, B. Maggio, D. Raffa, S. Plescia, M. L. Bajardi, A. Caruso, V. M. C. Cutuli, and M. Amico-Roxas, *Eur. J. Med. Chem*., 1994, **29**, 707.
16. N. Gokhan-Kelekci, S. Yabanoglu, E. Kupeli, U. Salgin, O. Ozgen, G. Ucar, E. Yesilada, E. Kendi, A. Yesilada, and A. A. Bilgin, *Bioorg. Med. Chem*., 2007, **15**, 5775.

17. I. Koca, A. Ozgur, K. A. Coskun, and Y. Tutar, *Bioorg. Med. Chem*., 2013, **21**, 3859.
18. K. M. Dawood, T. M. A. Eldebss, H. S. A. El-Zahabi, M. H. Yousef, and P. Metz, *Eur. J. Med. Chem*., 2013, **70**, 740.
19. L. Bhat, B. Jandeleit, T. M. Dias, T. L. Moors, and M. A. Gallop, *Bioorg. Med. Chem. Lett*., 2005, **15**, 85.
20. M. J. Naim, O. Alam, M. Farah Nawaz, J. Alam, and P. Alam, *J. Pharm. Bioallied Sci*., 2016, **8**, 2.
21. S. Vyas, K. Parmar, B. Patel, M. Chaudhari, N. Umrigar, and K. Goswami, *Orient. J. Chem*., 2022, **38**, 1064.
22. M. Marinescu, *Antibiotics*, 2021, **10**, 1002.
23. M. A. Alam., *Future Med. Chem*., 2022, **14**, 343.
24. O. Kulinkovich, N. Masalov, and V. Tyvorskii, *Tetrahedron*, 1996, **37**, 1095.
25. V. Kumar, K. Kaur, G. K. Gupta, and A. K. Sharmaet, *Eur. J. Med. Chem*., 2013, **69**, 735.
26. V. Kumar, K. Kaur, G. K. Gupta, and S. Kumar, *Recent Pat. Inflamm. Allergy Drug Discov*., 2013, **7**, 124.
27. P. Torres, A. Sodero, P. Jofily, and F. Silva-Jr, *Int. J. Mol. Sci*., 2019, **20**, 4574.
28. D. Filimonov, A. Rudik, A. Dmitriev, and V. Poroikov, *Int. J. Mol. Sci*., 2020, **21**, 7492.
29. M. A. Kira, M. O. Abdel-Raeman, and K. Z. Gadalla, *Tetrahedron Lett*., 1969, **2**, 109.
30. A. M. M. Shaker, E. K. A. Abdelall, K. R. A. Abdellatif, and H. M. Abdel-Rahman. *BMC Chem.*, 2020, **14**, 23.
31. R. A. Pawar and A. A. Patil, *Indian J. Chem*., 1994, **33B**, 156.
32. E. A. Palombo and S. J. Semple, *J. Ethnopharmacol*., 2001, **77**, 151.
33. M. X. Yang, X. Lin, P. Yu, L. J. Chen, and S. X. Liu, *Chin. J. Chem*., 2005, **23**, 1407.
34. NCCLS, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically M7-A5, National Committee on Clinical Laboratory Standards, Wayne, 2000, **20**, 2.
35. Y. K. Qi, B. Jiao, X. D. Ma, W. P. Cui, and S. T. Ma, *Arch. Pharm. Chem. Life Sci*., 2010, **343**, 458.