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Synthesis, Molecular Docking and *In Vitro* Antimicrobial Studies of New Hexahydroindazole Derivatives of Curcumin

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Abstract: A series of hexahydroindazole analogues of curcumin were synthesized and investigated for *in vitro* and *in silico* antimicrobial activity. The structures of synthesized compounds were identified on the basis of satisfactory analytical and spectral data (^{1}H NMR, ^{13}C NMR, EI-MASS techniques and elemental analysis). Synthesized compounds showed moderate to high activity against both bacterial and fungal strains. All compounds were docked computationally to the active site of enzyme L-glutamine: D-fructose-6-phosphate amido-transferase [GlcN-6-P] (EC 2.6.1.16). The autodock programme 4.0 was employed to perform automated molecular docking. *(E)-1-(7-(3-methoxybenzylidene)-3-(3-methoxyphenyl)-3,3a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone* (**A7**) turned out to be the most potent analogue of the series, showing best activity against bacterial and fungal strains. Compound **A7** showed minimum binding and docking energy and may be considered as good inhibitor of GlcN-6-P synthase. Further investigation and optimization of this lead could provide new antimicrobial molecules.

Keywords: Autodock 4.0; Bacterial strain; Fungal strains; GlcN-6-P synthase; Hexahydroindazole; *In silico*.

INTRODUCTION

In recent years, a number of antibiotics have lost their effectiveness due to the development of resistant strains. Extremely resistant bacteria such as methicillin-resistant *Styphlococoous aureus* (MRSA) and vancomycin-resistant Enterococci accounts for a soaring percentage of hospital acquired infections [1]. Multidrug resistance (MDR) to antibiotics presents a serious therapeutic problem in the treatment of bacterial infections. The importance of mechanism of resistance in clinical settings is reflected in increasing number of reports of multidrug resistant isolates [2]. In addition to this problem, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune-suppression allergic reactions etc. Therefore, there is a need to develop novel antimicrobial drugs for the treatment of infectious diseases. The search for not only the improved versions of existing drugs but also new drug targets have become an urgent need. Large number of marketed drugs have enzymes or membrane receptors as molecular targets [3]. In most cases, elucidating the intermolecular interactions in these drug-target complexes was a pre-requisite of the success [4].

The enzyme, L-glutamine:D-fructose-6-phosphateamido-transferase, known under the trivial name of glucosamine-6-phosphatesynthase (EC 2.6.1.16) is a new target for antimicrobial studies. The enzyme catalyses uridine 5'di-phospho-N-acetyl-D-glucosamine, the first and practically irreversible step in the pathway of hexosamine metabolism end-product, and serves as an activated form of *N*-acetyl-glucosamine. This amino sugar is incorporated into several macromolecules viz. peptidoglycan, lipopolysacc-harides and teichoic acids in bacteria, chitin in fungi, insects and crustaceans, and glycoproteins, glycosaminoglycans and mucopolysaccharides in mammals. All these molecules are essential for the assembly of the cell wall [5]. Several GlcN-6-P inhibitors, exhibiting antimicrobial activity have been reported, from both natural and synthetic origin.

Curcumin, a naturally occurring polyphenol is extracted from the rhizomes of *Curcuma longa*. It has been used as an important dietary component for long time. It exhibits various biological activities including antiproliferative activity against various cancer cells, antioxidant activity, wound healing ability and antimicrobial activity. Curcumin possesses antibacterial property against a number of Gram-positive and Gram-negative bacteria. It has been shown to kill several pathogenic Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus* that cause skin infections, pneumonia, meningitis and urinary tract infections in human being. Understanding the mechanism of the antibacterial activity of curcumin will greatly assist in designing potent analogues of curcumin, a natural remedy for several bacterial diseases [6-10].

Compounds containing hexahydroindazole are biologically active and have been reported as potent antimicrobial, anti-inflammatory, depressant of central nervous system and additionally some are also found to be monoamine oxidase inhibitors [11-12].

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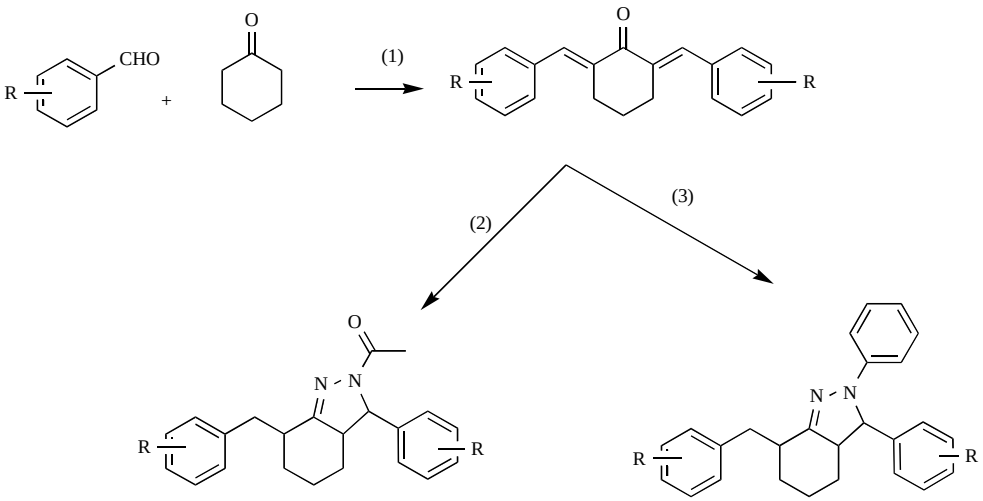


Scheme 1. Synthesis of hexahydroindazole derivatives of Curcumin (**A1-A9**) and (**B1-B5**). Reagents and conditions: (1) 10 % NaOH, EtOH, H_2O; (2) Hydrazine hydrate, Glacial acetic acid, 12 h reflux; (3) Phenyl hydrazine, Glacial acetic acid, 12 h reflux.

Minu *et al.* reported synthesis and antimicrobial activity of 2,3-disubstituted-3,3a,4,5,6,7-hexahydro-2H-indazole derivatives. They observed that the compounds with electron withdrawing groups were generally more active than other derivatives. Among the electron withdrawing halogen groups, presence of *p*-flurophenyl group at third position of hexahydroindazole improved the antibacterial activity. Whereas, the presence of *p*-chlorophenyl group at third position of hexahydroindazole improved the antifungal activity [13].

Synthesis of furfurylidene containing hexahydroindazoles with different pharmacophore fragments (furan and pyrazoline cycles, nitro-, azomethine, and other groups) was reported by Golikov *et al.* They observed that nitrofuran cycle linked to the pyrazoline ring containing azomethine group via avinilydene unit was most potent against gram positive bacteria. While, other substitutions (replacement of nitro group in furan by a methyl group or hydrogen) to hexahydroindazole resulted in diminished activity against all the tested organisms [14].

Present study includes the synthesis, molecular docking and antimicrobial studies of some novel hexahydroindazole derivatives of curcumin and attempt has been made to ascertain the role of various substituents on aryl ring with respect to the antibacterial activity.

MATERIALS AND METHODS

All reagents were obtained from commercial suppliers and were used without further purification. Reaction progress was monitored by thin layer chromatography (TLC) on pre-coated Merck alufoil plates (silica gel 60F-254, 0.25 mm thickness). Melting points were determined on a Veego capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker 300 MHz spectrophotometer. All NMR spectra were obtained in deuterated chloroform ($CDCl_3$); chemical shifts are reported in parts per million, and coupling constants in hertz (Hz). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet). Mass spectra were recorded on a LC-MS-2010A spectrometer and mass values are reported in m/z. Elemental analysis of synthesized compounds were recorded on EXETER-CE-440 elemental analyzer.

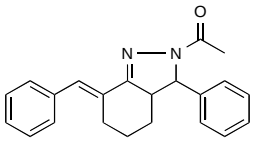
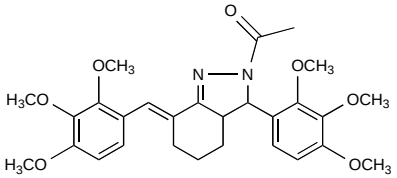
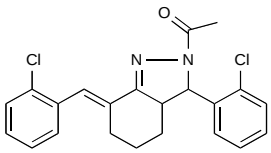
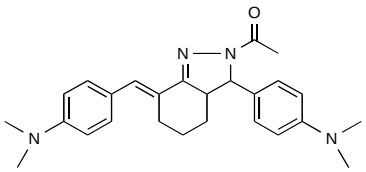
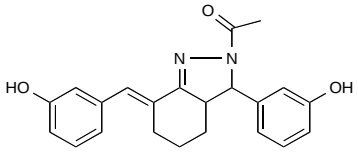
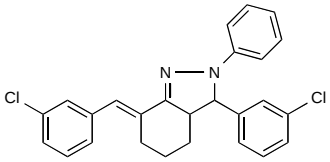
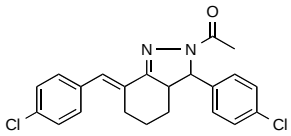
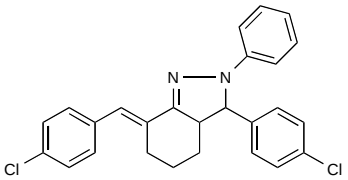
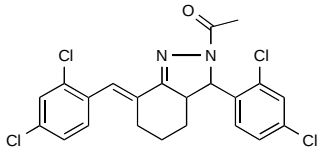
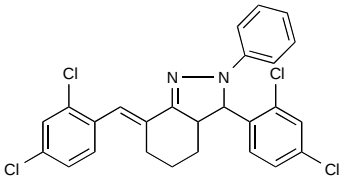
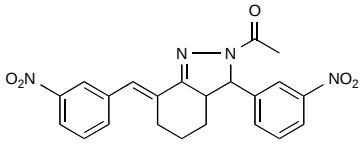
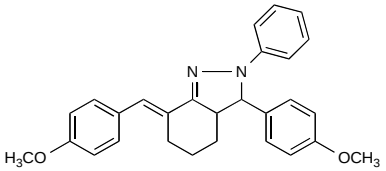
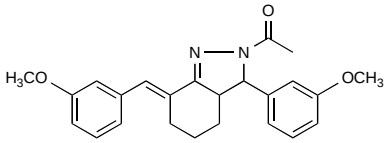
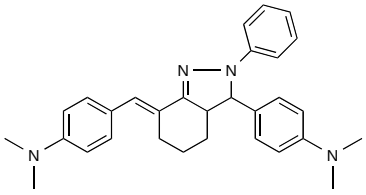
Molecular Docking Studies

Automated docking was used to determine the orientation of inhibitors bound to the active site of GlcN-6-P synthase. A genetic algorithm method, implemented in the program AutoDock 4.0, was employed [15]. The 3D structure file of all the curcumin analogues and fluconazole molecule were loaded on to PRODRG server [16] and PreADMET server for energy minimization and drug likeliness prediction respectively. The protein structure file 1Jxa was downloaded from Protein Data Bank (www.rcsb.org/pdb) was edited by removing the hetero atoms, adding C-terminal oxygen [17]. For docking calculations, Gasteigere-Marsili partial charges [18] were assigned to the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map was centered at the residues of the protein predicted from the CASTp server [19]. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization using default parameters. The number of docking runs was 50, the population in the genetic algorithm was 250, the number of energy evaluations was 100,000, and the maximum number of iterations was 10,000. The docking results for ligand molecules against glucosamine-6-phosphate synthase [PDB Id: 1jka], showed minimum docking energy, binding energy, inhibition constant, intermolecular energy with 0.0 RMS as documented. Computer with Microsoft Windows XP operating system, Intel Pentium 3.40 GHz processor, 1 GB RAM and Hard disk: 500 GB, Python: 2.4 was used.

Synthesis

The compounds were prepared by coupling substituted benzaldehydes with cyclohexanone (2:1) in a base catalyzed Claisen-Schmidt condensation followed by reflux with hydrazines (hydrazine hydrate, phenyl-hydrazine) and acetic acid (scheme **1**, Table **1**).

Table 1. Different substitutions on aryl ring of synthesized compounds.

H	A1		2,3,4 triOCH$_3$	A8	
2-Cl	A2		4-N(CH$_3$)$_2$	A9	
3-OH	A3		3-Cl	B1	
4-Cl	A4		4-Cl	B2	
2,4-diCl	A5		2,4-diCl	B3	
3-NO$_2$	A6		4-OCH3	B4	
3-OCH$_3$	A7		4-N(CH$_3$)$_2$	B5	

Synthesis of E-1-(7-(substituted bezylidene)-3-(substituted phenyl)-3,3a,4,5,6,7-hexahydro 2 H indazole-2yl) Ethanones (A1-A9)

These were prepared in two steps. In First step, respective dibenzylidiene cyclohexanones were synthesized. A mixture of ethanol (20ml) and sodium hydroxide solution (10%, 10 ml) was taken in a beaker, maintained at temperature between 15 to 25ºC. The solution was vigorously stirred and one half of previously prepared mixture of appropriate aromatic aldehyde and cyclohexanone (2:1) was added to it. After 20 min, the remaining of the aromatic aldehyde-cyclohexanone mixture was added. The reaction mixture was further stirred for 45 minutes. The solid separated was filtered off, washed with distilled water, and subjected to further purification by recrystallization with ethyl alcohol.

In second step, synthesized substituted dibenzylidene cycloheaxanone (1.2 mmol) from first step was dissolved in glacial acetic acid (5 mL), and hydrazine hydrate (1.5 mmol) was added to the solution. The solution was refluxed for 12 hours and reaction was monitored by TLC .The solvent was removed in vacuum and the residue was recrystallized with ethyl alchol. All the derivatives were also prepared by the same procedure.

(E)-1-(7-benzylidene-3-phenyl-3,3a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A1):

Yellow powder, mp 90-92^{0}C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.61-7.25 (m, 10H), 6.31 (s, 1H, =CH), 4.72 (d, 1H), 2.67 (m, 1H), 2.12 (s, 3H, CH_3), 1.81-1.42 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 166.9, 154.4, 141.7, 136.0, 134.9, 131.9, 128.5, 127.6, 127.1, 127.9, 126.3, 70.2, 41.8, 27.5, 24.8, 23.7. ESI-MS: m/z: (M^+, %) 330.14 (M^+ 6%), 329(97%), 200(100%). Elemental analysis found (Calc.) for $C_{22}H_{22}N_2O$: C, 79.97 (79.65); H, 6.71 (6.73); N, 8.48(8.45).

(E)-1-(7-(2-chlorobenzylidene)-3-(2-chlorophenyl)-3,3a,4,-5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A2):

Yellow powder, mp 102-104^{0}C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.72-7.20 (m, 8H), 6.69 (s, 1H, =CH), 4.5 (d, 1H), 2.67 (m, 1H), 2.02 (s, 3H, CH_3), 1.85-1.43 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 166.5, 154.6, 141.5, 136.5 134.7, 133.5, 133.1, 130.7, 129.5, 129.3, 129.3, 128.2, 127.1, 127.0, 126.6, 126.4, 65.9, 42.4, 23.3. ESI-MS: m/z: (M^+, %) 398.07. (M^+ 9%), 397(22%), 220(100%). Elemental analysis found (Calc.) for $C_{22}H_{20}Cl_2N_2O$: C, 66.17(66.43); H, 5.05(5.03); N, 7.02(7.30).

(E)-1-(7-(3-hydroxybenzylidene)-3-(3-hydroxyphenyl)-3,-3a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A3):

Yellow powder, mp 166-168^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.52-6.51 (m, 8 H), 6.31 (s, 1H, =CH), 5.30 (s, 2H), 4.6 (d, 1H), 2.55 (m, 1H), 2.32 (s, 3H, CH_3), 1.75-1.37 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 167.2, 158.9, 156.6, 155.5, 140.7, 135.6, 135.1, 131.7, 130.4, 129.7, 121.8, 120.4, 115.6, 113.4, 113.2, 22.2. ESI-MS: m/z: (M^+, %) 362 (M^+ 7%),361(96%), 221(100%). Elemental analysis found (Calc.) for $C_{22}H_{22}N_2O_3$: C, 72.91(72.71); H,6.12 (6.09); N, 7.73(7.76).

(E)-1-(7-(4-chlorobenzylidene)-3-(4-chlorophenyl)-3,3a,4,-5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A4):

Yellow powder, mp 126-128^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.66-7.58 (m,8 H), 6.27 (s, 1H, =CH), 4.2 (d, 1H), 2.65 (m, 1H), 2.01 (s, 3H, CH_3), 1.77-1.45 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 168.5, 155.6, 139.7, 136.0, 133.3, 131.5, 129.0, 128.6, 128.4, 70.9, 42.8, 27.8, 24.5, 21.4. ESI-MS: m/z: (M^+,%) 398 (M^+ 12%), 397(47%),229(100%). Elemental analysis found (Calc.) for $C_{22}H_{20}$ $Cl_2N_2O_2$: C, 63.62(63.36); H,4.85 (4.86); N,6.75 (6.77).

(E)-1-(7-(2,4-dichlorobenzylidene)-3-(2,4-dichlorophenyl)-3,3a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A5):

Yellow powder, mp 152-154^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.71-7.02 (m, 6H), 6.56 (s, 1H, =CH), 4.7 (d, 1H), 2.66 (m, 1H), 2.02 (s, 3H, CH_3), 1.85-1.23 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 163.5, 152.6, 150.1, 137.6, 136.8, 132.4, 132.1, 131.4, 131.0, 130.3, 127.9, 126.8, 126.5, 125.2, 120.8, 75.8, 42.1, 27.9, 24.3, 23.1. ESI-MS: m/z: (M^+, %) 468(M^+ 12%), 467(22%), 221(100%). Elemental analysis found (Calc.) for $C_{22}H_{18}$ Cl_4N_2O : C, 56.44(56.66); H,3.88 (3.83); N, 5.98 (5.96).

(E)-1-(7-(3-nitrobenzylidene)-3-(3-nitrophenyl)-3,3a,4,5,6-,7-hexahydro-2H-indazol-2-yl) ethanone (A6):

Yellow powder, mp 130-132^0 C. ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 8.29-7.54 (m, 8H), 6.42 (s, 1H, =CH), 4.4 (d, 1H), 2.63 (m, 1H), 2.02 (s, 3H, CH_3), 1.81-1.43 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 167.5, 154.6, 146.8, 146.2, 140.4, 136.1, 135.3, 134.6, 134.2, 129.5, 129.3, 128.7, 123.8, 122.7, 121.7, 120.7, 69.7, 42.8, 27.8, 24.5, 23.4. ESI-MS: m/z: 420(M^+ 12%),.418(100%). Elemental analysis found (Calc.) for $C_{22}H_{20}$ N_4O_5 : C, 62.85(62.60); H,4.79 (4.80); N, 13.33(13.28) .

(E)-1-(7-(3-methoxybenzylidene)-3-(3-methoxyphenyl)-3,3-a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A7):

Yellow powder, mp 110-112^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.49-6.21 (m,8 H), 6.11 (s, 1H, =CH), 4.1 (d, 1H), 3.75 (s, 6H), 2.68 (m, 1H), 2.04 (s, 3H, CH_3), 1.78 -1.32 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 169.5, 161.5, 160.4, 155.6, 142.5, 136.0, 134.8, 128.6, 126.5, 120.8, 120.4, 112.5, 112.0, 111.5, 71.2, 55.8, 42.8, 27.1, 24.5, 23.4. ESI-MS: m/z: 390((M^+ 8%), 389(26%), 212(100%). Elemental analysis found (Calc.) for $C_{24}H_{26}N_2O_3$: C, 73.82(73.63); H, 6.71(6.72); N, 7.17(7.16).

(E)-1-(7-(2,3,4-trimethoxybenzylidene)-3-(2,3,4-trimethoxy-phenyl)-3,3a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A8):

Yellow powder, mp 210-212^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.47 -6.25 (m,4 H), 6.59 (s, 1H, =CH), 4.4 (d, 1H), 3.75 (s, 9H), 2.68 (m, 1H), 2.04 (s, 3H, CH_3), 1.80-1.35 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 168.5, 156.8, 155.9, 152.9, 151.1, 149.1, 142.3, 141.6, 136.0, 135.7, 132.7, 122.7, 121.4, 119.6, 108.2, 106.8, 104.7, 65.3, 61.7, 60.8, 60.3, 56.1, 43.1, 27.8, 24.5, 23.4. ESI-MS: m/z: 510(M^+ 12%), 507(20%), 215(100%). Elemental analysis found (Calc.) for $C_{28}H_{34}N_2O_7$: C, 65.87(65.92); H, 6.71(6.70); N, 5.49(5.48).

(E)-1-(7-(4-(dimethylamino)benzylidene)-3-(4-(dimethyla-mino)phenyl)-3,3a,4,5,6,7-hexahydro-2H-indazol-2-yl)ethanone (A9) :

Yellow powder, mp 150-152^0 C.^{1}H NMR (CDCl3, 300 MHz, 25 °C): 7.64 -6.56 (m,10 H), 6.25 (s, 1H, =CH), 4.7 (d, 1H), 3.78 (s, 12 H), 2.68 (m, 1H), 2.54 (s, 3H, CH_3), 1.81-1.36 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 167.5, 154.6, 151.3, 149.3, 133.7, 132.1, 131.4, 128.7, 124.7, 112.7, 111.7, 72.9, 41.3, 26.8, 24.5, 23.4. ESI-MS: m/z: 416(M^+ 9 %), 415(21%), 221(100%). Elemental analysis found (Calc.) for $C_{26}H_{32}N_4O$: C, 74.97(74.96); H, 7.74(7.72); N, 13.45(13.43).

Synthesis of E-7-(substituted benzylidene)-2-phenyl-3-(substituted phenyl)-3, 3a, 4,5,6,7 hexahydro 2H indazoles (B1-B5)

In the first step, the dibenzylidene cycloheaxanones were synthesized as reported. The synthesized dibenzylidene cycloheaxanon(1.2 mmol) from first step was dissolved in glacial acetic acid (5 mL) and phenyl hydrazine (1.5mml) was added to the solution. The solution was refluxed for 12 hours and monitored by TLC. Solvent was removed in vacuum and recrystallized with ethlyl alcohol. The

Table 2. Antimicrobial activity of new hexahydroindazole derivatives of curcumin.

Comp no.	Bacterial Strains Zone of inhibition (in mm)					Fungal Strains Zone of inhibition (in mm)		
	E.coli ATCC 35218	*S. typhi* MTCC 3216	*K. pneumonia*	*S. aureus* ATCC 25323	*E. faecalis*	*C. albicans* ATCC 90028	*C. tropicalis* ATCC 750	*C. krusie*
A1	NA	NA	NA	10.37±0.38	9.70±0.36	NA	NA	NA
A2	15.60±0.26	14.37±0.12	14.87±0.15	13.20±0.17	15.83±0.19	14.80±0.25	12.53±0.18	12.83±0.15
A3	13.30±0.25	14.93±0.18	17.63±0.09	16.77±0.03	14.57±0.15	16.27±0.23	11.30±0.15	11.20±0.15
A4	9.40±0.12	NA	NA	11.73±0.38	10.50±0.21	13.37±0.09	NA	NA
A5	8.93±0.09	NA	10.27±0.09	11.70±0.12	NA	12.67±0.09	NA	NA
A6	8.07±0.09	NA	NA	10.23±0.15	9.03±0.12	NA	NA	NA
A7	21.43±0.18	17.20±0.17	20.40±0.11	22.53±0.09	20.33±0.15	23.0±0.12	21.50±0.12	19.77±0.15
A8	19.67±0.11	15.10±0.15	16.80±0.17	17.0±0.16	15.23±0.20	20.33±0.12	18.20±0.17	17.87±0.19
A9	10.90±0.06	NA	NA	10.03±0.18	12.10±0.20	11.93±0.09	NA	12.47±0.07
B1	13.93±0.09	NA	8.87±0.13	7.10±0.17	6.63±0.09	7.63±0.05	5.73±0.12	NA
B2	8.07±0.06	NA	NA	6.77±0.15	4.07±0.18	NA	NA	NA
B3	7.87±0.10	NA	NA	NA	NA	6.70±0.13	NA	5.93±0.09
B4	9.33±0.07	8.33±0.09	NA	5.57±0.12	4.87±0.09	NA	NA	NA
B5	8.90±0.08	NA	NA	NA	4.17±0.15	NA	NA	3.30±0.17
Cip	26.23±0.16	19.98±0.06	26.71±0.08	27.44±0.15	22.50±0.09	NA	NA	NA
Fluc	NA	NA	NA	NA	NA	25.33±0.05	24.65±0.05	24.06±0.04

The value of each compound consisted of Mean ± SE of 03 replicates.
Level of significance $p < 0.05$

remaining derivatives were also prepared by the same procedure.

(E)-7-(3-chlorobenzylidene)-3-(3-chlorophenyl)-2-phenyl-3,3a,4,5,6,7-hexahydro-2H-indazole (B1):

Yellow powder, mp 58-60^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.34-6.75 (m, 13 H), 6.24 (s, 1H, =CH), 3.7 (d, 1H), 2.25 (m, 1H), 1.78-1.43 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 145.6, 143.8, 140.9, 136.0, 134.9, 134.2, 134.1, 130.0, 129.9, 129.5, 128.0, 126.9, 126.2, 119.7, 62.6, 40.5, 26.8, 24.8, 23.5. ESI-MS: m/z: 432(M^+ 7%), 430(100%). Elemental analysis found (Calc.) for $C_{26}H_{22}Cl_2N_2$: C, 72.06(71.97); H, 5.12(5.11); N, 6.46(6.52).

(E)-7-(4-chlorobenzylidene)-3-(4-chlorophenyl)-2-phenyl-3,3a,4,5,6,7-hexahydro-2H-indazole (B2):

Yellow powder, mp 78-80^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.78-6.73 (m, 13 H), 6.23 (s, 1H, =CH), 3.5 (d, 1H), 2.26 (m, 1H), 1.82-1.41 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 154.6, 143.8, 138.6, 136.0, 133.5, 133.3, 131.7, 131.5, 129.0, 129.5, 128.6, 128.4, 116.7, 63.1, 42.9, 28.6, 27.8, 24.8, 24.5. ESI-MS: m/z: 432(M^+ 6%), 430(100%). Elemental analysis found (Calc.) for $C_{28}H_{24}Cl_2N_2$: C, 73.20(73.37); H, 5.27(5.28); N, 6.10(6.11).

(E)-7-(2,4-dichlorobenzylidene)-3-(2,4-dichlorophenyl)-2-phenyl-3,3a,4,5,6,7-hexahydro-2H-indazole (B3):

Yellow powder, mp 70-72^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.58-6.75 (m, 13H), 6.59 (s, 1H, =CH), 3.8 (d, 1H), 2.27 (m, 1H), 1.87-1.30 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 154.6, 150.5, 141.8, 138.6, 136.0, 133.8, 133.1, 131.7, 131.1, 130.3, 129.4, 128.9, 128.1, 126.7, 120.8, 116.7, 58.0, 41.4, 28.7, 24.5. ESI-MS: m/z: 502(M^+ 3%), 501(15%), 225(100%). Elemental analysis found (Calc.) for $C_{26}H_{20}Cl_4N_2$: C, 62.17(62.31); H, 4.01(4.02); N, 5.58(5.56).

(E)-7-(4-methoxybenzylidene)-3-(4-methoxyphenyl)-2-phenyl-3,3a,4,5,6,7-hexahydro-2H-indazole (B4):

Yellow powder, mp 80-82^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.52-6.84 (m,8 H), 6.32 (s, 1H, =CH), 3.5 (d, 1H), 3.73 (S, 6H), 2.25 (m, 1H), 1.84-1.42 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 158.8, 156.8, 144.8, 134.0, 131.8, 131.2, 130.2, 129.5, 127.8, 127.5, 121.8, 116.7, 113.2, 112.1, 63.1, 55.8, 42.9, 27.8, 24.8, 24.5. ESI-MS: m/z: 424(M^+ 4%), 215(100%). Elemental analysis found (Calc.) for $C_{28}H_{28}N_2O_2$: C, 79.22(79.43); H, 6.65(6.66); N, 6.60(6.62).

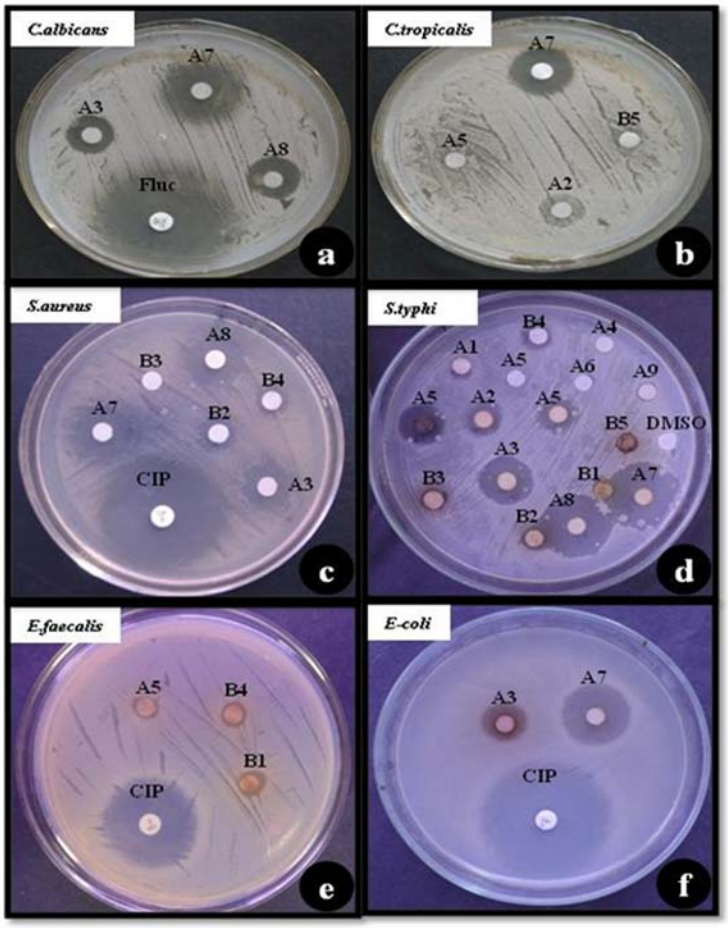


Fig. (1). Antimicrobial activity of hexahydroindazole derivatives of Curcumin **(a)** antifungal activity against *candida albicans* with refrence to standard drug Fluconazole, A7 showing the strongest inhibition compare to A3 and A8 **(b)** antifungal activity against *C.tropicalis*, A7 showing clear zone of inhibition as compare to A5, B5 and A2 **(c)** Antibacterial against *S.aureus* with reference to standard drug Ciprofloxacin, A3, A8 and A7 showing inhibition much better than B3, B2,and B4 **(d)** antibacterial against *S.typhi*, A3, A5, A8, A7, A2, A5 and B4 showing inhibition zone **(e)** antibacterial against *E.faecalis* with reference to Ciprofloxacin as standard, A5, B4, and B1 showing less inhition compare to standard **(f)** antibacterial activity against *E-coli*, A7 and A3 showing good inhibition as compared to standard Ciprofloxacin.

(E)-4-(7-(4-(dimethylamino) benzylidene)-2-phenyl-3, 3a, 4, 5, 6, 7-hexahydro-2H-indazol-3-yl)-N,N-dimethylaniline (B5):

Yellow powder, mp 178- 179^0 C.^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): 7.62-7.01 (m, 13 H), 6.24 (s, 1H, =CH), 3.1 (d, 1H), 3.07 (s, 12 H) 2.31 (m, 1H), 1.78 -1.20 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): 154.2, 150.1, 146.3, 143.8, 136.7, 130.7, 130.4, 130.0, 129.7, 129.5, 128.9, 128.4, 127.1, 126.9, 116.7, 112.7, 111.7, 63.1, 42.9, 40.3, 27.8, 24.8, 24.5. ESI-MS: m/z: 450(M^+ 3%), 135(100%). Elemental analysis found (Calc.) for $C_{30}H_{34}N_4$: C, 79.96(80.02); H, 7.61(7.62); N, 12.43(12.41).

Antimicrobial Studies

Antimicrobial activities of newly synthesized compounds were first screened by disc diffusion method [20] against various Gram positive and Gram negative human pathogenic bacteria viz. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27893, *S. typhi* MTCC 3216, *E. faecalis* and *Staphylococcus aureus* ATCC 25323 and different fungal strains of *Candida* according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS, 1997).

Freshly grown microbial strains were mixed in sterile saline (0.85%) and the turbidity was matched with McFarland No. 2 system to achieve concentration of 107 CFU/ml. Sterile petri plates containing 20 mL of Mueller Hinton agar (MHA, Hi-Media) were used for all bacterial culture and Sabouraud's dextrose agar (SDA)/Potato dextrose agar (PDA) (Hi-Media) were used for all fungal culture. The bacterial inoculums' suspension was spread on the surface of agar plates. Sterile disc (5mm) of Whatman paper no. 1 was then placed on the surface of the media and the test compounds (25µl/ml) were put and allowed to diffuse and plates were incubated for 24 h at 37ºC for bacterial cultures and 72 h at 25 ºC for fungal culture. Ciprofloxacin (5µg/disc, Hi-Media) was used as positive control for bacteria and Fluconazole (10µg/disc, Hi-Media), was used as a positive control for fungi. Zone of

Table 3. Theoretical prediction of different properties of new hexahydroindazole derivatives of curcumin using PreADMET Server.

Entry	Log_p	Human intestinal Absorption (%)	*In vitro* skin permeability (logKp, cm/hour)	*In vitro* plasma protein binding (%)	Water solubility in buffer system Mg/L
A1	4.62	99.54	-2.45	93.96	403.4
A2	5.70	99.68	-2.42	94.26	45.21
A3	3.61	94.30	-2.95	90.87	207.1
A4	5.98	99.68	-2.45	100	144.0
A5	7.01	99.59	-2.27	100	5.57
A6	3.69	93.90	-2.42	93.48	5.53
A7	4.69	97.37	-2.82	89.91	128.1
A8	4.10	98.91	-3.40	87.51	15.57
A9	4.82	98.31	-2.52	92.61	55.88
B1	8.23	100	-1.77	100	$5.37x10^{-6}$
B2	8.27	100	-1.76	100	$1.50x10^{-5}$
B3	8.85	100	-1.69	100	$5.77x10^{-7}$
B4	7.07	99.32	-2.05	96.34	$3.77x10^{-5}$
B5	7.10	100	-1.78	93.07	$5.83x10^{-6}$

Table 4. Molecular docking of new hexahydroindazole derivatives of curcumin with glucosamine-6-phosphate synthase.

Entry	Binding Energy (Kcal/mol)	Docking Energy (Kcal/mol)	Inhibition constant (M)	Amino acid residue involved in H-bond	Bond Length (Å)	RMSD
A1	-9.25	-10.03	$1.67x10^{-7}$	Arg 73:HH1::Dp1:NA	1.67	0.81
				His 77:HE2::Dp1:NA	2.18	
A2	-9.99	-10.78	$4.79x10^{-8}$	Arg 73:HE::Dp2:OA	2.05	0.92
				Arg 73:HH4::Dp2:NA	2.08	
A3	-9.57	-10.28	$9.63x10^{-8}$	Gly 99:HN::Dp3:OA	1.90	0.99
A4	-8.19	-8.54	$9.97x10^{-7}$	No H- Bonds	--	1.12
A5	-9.56	-9.64	$9.80x10^{-8}$	No H- Bonds	--	1.20
A6	--	--	--	--	--	
A7	-10.02	-10.96	$4.50x10^{-8}$	Gly 99:HN::Dp7:OA	2.00	0.56
A8	-6.93	-8.48	$8.57x10^{-6}$	Arg 73:HH21::Dp8:OA	1.68	1.02
				His 77:HE2::Dp8:OA	2.13	
A9	-5.40	-4.86	$1.10x10^{-4}$	His 71:HE2::Dp9:NB	1.88	1.11
B1	-7.64	-8.35	$2.49x10^{-6}$	His 77:HE2::Dp10:NA	2.01	0.96
B2	-7.51	-8.40	$3.14x10^{-6}$	No H- Bonds	--	1.23
B3	-8.69	-9.07	$4.29x10^{-7}$	No H- Bonds	--	1.20
B4	-8.53	-8.40	$5.59x10^{-7}$	Cys 1:SG::Dp13:OA	2.93	0.95
B5	-7.12	-7.55	$6.05x10^{-6}$	No H- Bonds	--	1.23
Fluconazole	-5.81	-5.36	$5.53x10^{-5}$	Arg 73:HH21::DF:OA	2.20	1.97
				Arg 73:HE::DF:OA	2.09	
				His 77:HE2::Dp10:NA	1.87	

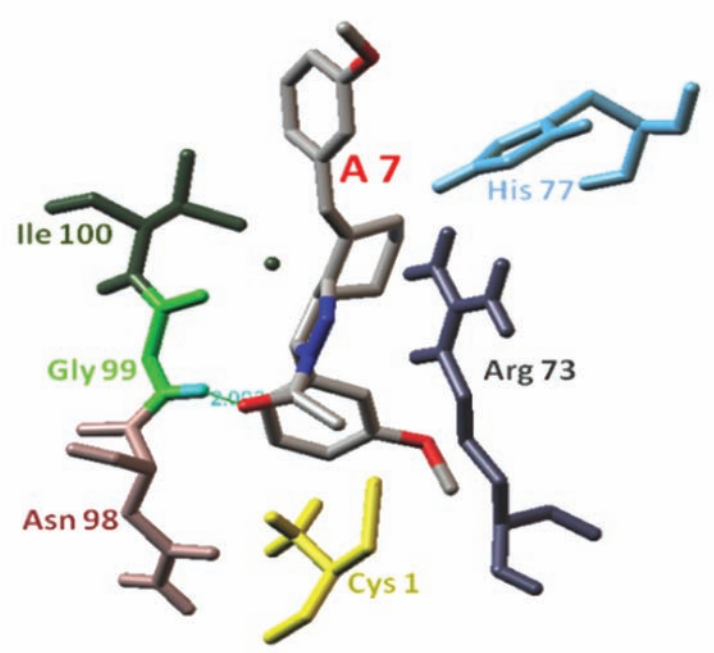


Fig. (2). Interaction of compound A7 with GlcN-6-P.

inhibition was measured in millimeters after 24 h. All tests were performed in triplicate.

RESULT AND DISCUSSION

The compounds were prepared by coupling substituted benzaldehydes with cyclohexanone in (ratio of 2:1) in a base catalyzed Claisen-Schmidt condensation followed by reflux with hydrazines (hydrazine hydrate, phenyl-hydrazine) and acetic acid. The results of antimicrobial screening of synthesized hexahydroindazole derivatives of curcumin are presented in table (Table **2**).

The results of antibacterial screening revealed that among the compounds screened compounds **A2**, **A3**, **B1** and **B2** showed moderate antibacterial activity while compound **A7** & **A8** displayed good antibacterial activity when compared with ciprofloxacin used as standard. Particularly, compound **A7** which is carrying methoxy group on aryl ring appears to exhibit maximum antibacterial activity (zone of inhibition up to 21 mm) against *E. coli* and *S. aureus*. Interestingly, triple substitution of methoxy group at ortho, meta and para positions leads to a slight decrease in the potency (compound A8 zone of inhibition = 19.67mm), this may be due to the steric hindrance by these groups. Electron donating group (methoxy) significantly increases antibacterial activity.

Derivatization of **B1** with chloro substitution on phenyl ring enhanced the potency (Compound **B1** zone of inhibition = 13.93mm) indicating that substitutions may result in restoration of potency. Whereas, methoxy (Compound **B4** zone of inhibition = 9.33mm) and N, N-dimethylamino (B5, zone of inhibition = 8.90mm) substitutions exhibited moderate potency. Phenyl pyrazoline analogues (**B1-B5**) did not significantly increase the antibacterial potency.

The results of antifungal screening indicated that compounds, **A2**, **A3**, **A4**, **A7** and **A8** showed moderate to excellent antifungal activity. Compounds **A2**, **A3** and **A4** showed moderate activity against *C. albicans*. Interestingly, compound **A3** also showed moderate activity against three fungal strains viz. *C. albicans*, *C. tropicalis*, *C. krusie*. Among the halogenated derivatives chloro substitution at ortho or para position increased antifungal potency appreciably (compound **A2** zone of inhibition = 14.80mm), (compound **A4** zone of inhibition = 13.37mm) Fig. (**1**).

Chloro substitution at both ortho and para positions resulted in marginal decrease in potency (compound **A5** zone of inhibition = 12.67mm). This may be attributed to successful interaction of the compound with target protein(s) when chloro substitution occurred either at ortho or para position. Out of 14 compounds synthesized, majority of them showed considerable antimicrobial activity against tested strains.

The combinatorial chemistry and virtual screening have rapidly increased in drug discovery. Need for minimizing extremely time-consuming steps of synthesis, biological screening and good tools for predicting drug-likeness is very much desired. The molecular descriptors have been employed to predict various human ADMET processes and other pharmacokinetic parameters such as oral absorption, bioavailability, skin penetration, clearance, volume of distribution, and metabolism [21-22]. Predicting ADME properties at an early stage of drug discovery and development process is very important to remove compounds with poor pharmacokinetic properties and minimize extremely expensive and time-consuming steps. Different properties viz. drug-likeliness, Human intestinal absorption, *In vitro* skin permeability, *in vitro* plasma protein binding and water solubility in buffer system of all the 14 synthesized hexahydroindazole analogues of curcumin were determined using PreADMET server. The results are summarized in table (Table **3)**.

Comparative docking of glucosamine-6-phosphate synthase (GlcN-6-P synthase) protein with the curcumin analogues and the standard drug fluconazole and

amphotericin B yielded best possible conformations with parameters including the binding energy, docked energy, inhibition constant and RMSD (Table **4**). Theoretically, molecules showed very good binding energy and docking energy ranging from -5.40 Kcal/mol to -10.02 Kcal/mol and -4.86 Kcal/mol to -10.96 Kcal/mol respectively Fig. (**2**). The minimum docked energy was found in the analogue A7 (-10.96 kcal/mol) with an estimated inhibition constant of $4.50x10^{-8}$ and RMSD 0.56. Whereas, docked energy of the standard drugs fluconazole was -5.67 kcal/mol with an inhibition constant of $5.53x10^{-5}$ and RMSD 1.97.

The accuracy of dockings was evaluated by the root-mean square deviation (RMSD) of docked ligand from original crystal structure. RMSD less than 1.0 Å was considered as excellent.

A significant number of drugs and drug candidates in clinical development are halogenated structures. The formation of halogen bonds in ligand-target complexes is recognized as a kind of intermolecular interaction that favorably contributes to the stability of protein-ligand complexes. The insertion of halogen atoms has been used in innumerous cases of hit-to-lead or lead-to-drug conversions [23-25].

The incorporation of halogen atoms increase membrane permeability, improve the oral absorption and skin penetration etc [26]. It is generally accepted that halogen atoms are not capable of significant hydrogen bonding. Six analogues containing chlorine as halogen atom (**A2**, **A4**, **A5**, **B2**, **B3** and **B5**) have not formed any hydrogen bond with the active site amino acids of GlcN-6-P synthase, but showed very good binding and docking energy, since the halogens are endowed with the ability to establish intermolecular bonds in a fashion that resembles the H-bonds.

In the present study, the molecular docking results of compound **A7** showed very good binding energy and RMSD, even in *in vitro* studies also **A7** emerged active against all tested microorganisms.So it can be predicted that the activity may be due to the inhibition of enzyme GlcN-6-Psynthase, which catalyses a complex reaction involving ammonia transfer from L-glutamine to Fru-6-P followed by isomerisation of the formed fructosamine-6-phosphate to glucosamine-6-phosphate.

CONCLUSION

This study allows us to conclude that enviable improvement of antimicrobial activities in synthesized compounds require electron donating groups and methoxy substitution may be the reason for highest activity of Compound **A7** among tested compounds. It is juvenile to arrive at the conclusion on structure activity aspect of these compounds and further assessment is desirable to use them for clinical study. Molecular docking studies also revealed that compound **A7** has minimum binding and docking energy and may be considered as good inhibitor of GlcN-6-P. The study may exalt the scope of developing these hexahydroindazole derivatives of curcumin as promising antibacterial and antifungal agents.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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