SYNTHESIS AND BIOLOGICAL EVALUATION OF SUBSTITUTED MANNICH BASES OF BENZOTRIAZOLE DERIVATIVES AS ANTICANCER AGENTS

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Abstract: Background: Benzotriazole-based compounds with various outstanding bioactivities have become increasingly active in the field of medicinal chemistry. Importantly, some anticancer benzotriazole compounds such as vorozole and TBB have been clinically used. It can be reasonable to expect that benzotriazole derivatives will play remarkable roles in medicinal field.

Methods: The *in silico* molecular docking and ADMET studies of the designed compounds were performed on binding cavity of Legumain using Schrodinger 2021-3. New mannich base derivatives of benzotriazoles (**6a-p**) were synthesized and these compounds were characterized by ^{1}HNMR, ^{13}CNMR and mass spectral data. These compounds (**6a-p**) were investigated for their anticancer properties towards four different human cancer cell lines by utilize of MTT method.

Results: The IFD results are in agreement with those of XP docking studies, confirming the binding of the test compounds in the binding site of legumain. The predicted ADMET properties of compounds fall within the acceptable range. Most of the compounds were displayed good to moderate anticancer activities to compare with control drug. The compounds showed IC_{50} valuesrange from 0.012±0.001 to 22.9±9.11 µM, and positive control showed values from 0.13 ± 0.017to 3.08 ± 0.135µM. Among synthesized compounds, these **6a, 6c, 6e, 6f**, **6j**, **6n** and **6p** were demonstrated more potent anticancer activities than etoposide.

Conclusion: Among all the synthesized compounds the compound **6a** contain electron-donating(3,5-dimethoxy) group on the phenyl ring displayed highest anticancer activity on four cancer cell lines with IC_{50} values as MCF-7=0.012±0.001µM, A549=0.18±0.076µM, Colo- 205=0.34±0.083µM and A2780= 0.07±0.006µM, respectively.

Keywords: MCF-7, A549, Colo-205, A2780, Benzotriazole, *In silico*, ADMET and MTT Assay

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INTRODUCTION

Benzotriazole-based compounds are unique nitrogen-containing heterocycles that have attracted significant attention from medicinal chemists as a promising class of bioactive heterocyclic products that exhibit numerous biological properties, such as anti- cancer, antibacterial, anti- inflammatory, antimalarial, analgesic, antifungal, and antitubercular activity [1]. Numerous proteins exhibit agonistic responses to benzotriazole derivatives. Apart from these uses, benzotriazole is a precursor in the synthesis of peptides, acid azides, and 3-hydroxymethyl-2,3- dihydrobenzofurans and 3-hydroxymethylbenzofurans [2]. Benzotriazole exists in three tautomers: two 1H-forms and one 2H-form. The equilibrium in solution favours the 1H-forms [4]. With a PKa of 8.2, benzotriazole is a very weak base, but a stronger NH-acid than 1,2,3-triazole, indazole, or benzimidazole[5-7]. According to the literature, benzotriazoles exhibit a variety of biological properties, including antiviral, antibacterial, anticonvulsant, antifungal, anti-inflammatory, and anticancer activity. In general, nitrogen and sulfur containing organic compounds and their metal complexes display a wide range of biological activity as antitumor, antibacterial, antifungal and antiviral agents [8]. Along with activities, benzotriazole is also a precursor in the synthesis of peptides, acid azides, preparation of 3hydroxymethyl- 2,3-dihydrobenzofurans and 3-N-Substituted benzotriazoles exist as two isomers: 1H- and 2H-substituted. It is generally agreed that 1H-substituted dominated in solid and solution, whereas the proportion of the 2H-tautomer increased in the gas phase [10]. However, the energy difference between the two isomers is very little [11]. Similarly, benzotriazoles containing Mannich bases have recently been synthesized also by amine exchange reactions, from the N, N-dimethyl amino propiophenone hydrochlorides and benzotriazole, respectively [12]. One of the most studied compounds of the triazole class is 1,2,3 - benzotriazole (BTA or BTAH1), $C_6H_5N_3$. The protective effect of BTA and its derivatives on various metals and

alloys have been attracting the attention of researchers for more than half a century [13]. Over the past decade, several interesting reviews have been devoted to them [14–20]. Alarge number of compounds containing 1, 2, 3-benzotriazole system have been investigated for broad spectrum of activities which include anti-corrosive[21], antiviral[22], anti-inflammatory, anticonvulsant[23], enzyme inhibitor[24], DNA cleavage[25], antifungal[26], herbicidal[27], Antitubercular[28], antimicrobial[29], anti- proliferative [30] etc. Furthermore, substitution of the 3,4,5-trimethoxyphenyl ring of isoCA-4 by a quinazoline nucleus [31] or quinoline [32-34] led to tubulin inhibitor with potent antiproliferative deeds versus a variety of cancerous cell lines.

Recently, BZII with a benzo- triazole moiety for the replacement for trimethoxyphenyl moiety of colchicine was reported, and the docking studies of BZII showed that benzotriazole formed polar and hydrophobic interaction with the critical residue amino acids of α- and β-tubulin subunits in the colchicine-binding pocket. These data demonstrated that benzotriazole moiety might be a surrogate of the traditional 3,4,5-trimethoylphenyl moiety when binding to the colchicine site. The ability of mankind to synthetically prepare medicinally important molecules during the past century has allowed for a continued decrease in the mortality rate from numerous diseases. Heterocyclic compounds containing nitrogen atoms are considered to be one of the most effective anti-microbial drugs used either as single agents or in combination for cancer therapy [35-36]. The biological target related to the anticancer activity of the compounds was predicted using target prediction tool in ChEMBL (https://www.ebi.ac.uk/chembl/). The most likely target for these compounds is Legumain, which is reported to be overexpressed in different types of cancers in humans. *In silico* molecular docking studies were performed to provide some insights on whether the binding of the compounds in the binding site of legumain is thermodynamically favourable and, if yes, the molecular interactions between the compounds and amino acid resides of the binding site. GLIDE searches favourable interactions between compound and the target protein. Based on these inspiring results, we have dedicated ourselves to designing and introducing novel anticancer agents. We proposed a chain of N-(1-(1H-Benzo[d][1,2,3] triazol-1-yl)alkyl)- substituted benzenamine (6a-6p) as novel heterocyclic analogs of benztriazoles by mannich base reaction. A new approach towards the development of a new series of novel benztriazoles derivatives was proposed by replacing the 1NH hydrogen with substituted aldehydes and substituted aromatic amines. In this paper we report on *in silico* design, their synthesis and potentantitumor activities versus human cancer cell lines.

MATERIALS AND METHODS

***In silico* Studies**

The designed hybrids were optimized preliminarily by the help of Chemdraw Professional 16. All the geometries of the ligands have been optimized by using Builder panel in Maestro v11.3 (v4.1, Schrodinger 2020-2)[37-38]. Ligprep 2020-2 was used as energetically minimize analogues for input structure. The optimized legumain (PDB ID: 5LUB) was prepared by using the protein preparation wizard (Epik v4.1, Schrodinger suite 2020-2)[37-38]. Before protein optimization, the water molecules were removed from the crystal structure. The docking study was performed as per the literature method by Glide Integrated Maestro 11.3[39]. The interaction and selectivity of the designed hybrids were observed for binding pocket of legumain.

ADMET Studies

QikProp is a fast and accurate tool available in Schrödinger for predicting the pharmaceutically relevant properties of organic compounds including absorption, distribution, metabolism, excretion, and toxicity (ADMET)[40]. It provides numerical values/range to compare a test compound's properties with those of 95% of known drugs.

Chemistry

Chemicals used were of reagent category and are purified as needed. Melting points were determined by Lab India digital melting point apparatus. Shimadzu FTIR spectrometer model used for recording IR spectrum of compound. Bruker DRX-300 spectrometer was used for determination of NMR spectra in DMSO solvent with internal standard as a TMS. Shimadzu LCMS 2010A spectrometer was used as examine a mass spectra of compounds. Responses were checked by thin layer chromatography (TLC).

General Procedure for the synthesis of N-(1-(1H-Benzo[d][1,2,3] triazol-1-yl)alkyl)- substituted benzenamine (6a-6p)

The mixture of benzotriazole (3) (1 g, 8.39 mmol) formaldehyde (4a) (3 ml, 10 mmol) and 3,5-dimethoxyaniline (5a) (1.3 g, 8.39 mmol) was stirred at room temperature for 1 hour in ethanol solvent. After completion of reaction by TLC, the reaction mixture was cooled to 25 °C and stirred for 5 hours followed by cooled at -5 °C for 16 hours (Figure 1). The resulting precipitate was filtered off, washed with diethyl ether and dried in vacuum to afford pure compounds 6a-6p with 46□-75% yield.

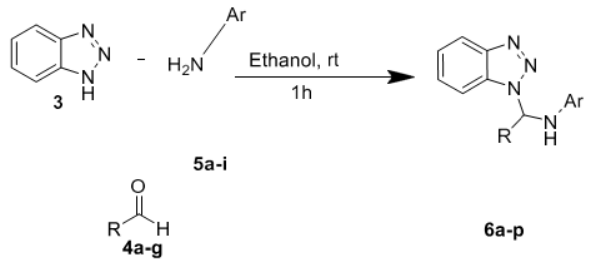

Figure 1. Scheme for the synthesis of N-(1-(1H-Benzo[d][1,2,3]triazol-1-yl)alkyl)-substituted benzenamine (6a-6p)

Table 1. List of derivatives with R and Ar substitutions

Compound	R	Ar
4a	H	-
4b	4-fluorophenyl	-
4c	4-(N,N'- dimethylamino)phenyl	-
4d	4-chlorophenyl	-
4e	3-chlorophenyl	-
4f	ethyl	-
4g	methyl	-
5a	-	3,5-dimethoxyphenyl
5b	-	4-chlorophenyl
5c	-	4-nitrophenyl
5d	-	3-chlorophenyl
5e	-	3,5-dinitrophenyl
5f	-	4-aminopyridyl
5g	-	3,5-dimethoxy-4- methylphenyl
5h	-	phenyl
5i	-	4-methoxyphenyl
6a	H	3,5-dimethoxyphenyl
6b	H	3,5-dinitrophenyl
6c	H	4-aminopyridyl
6d	H	3,5-dimethoxy-4- methylpheny
6e	ethyl	4-chlorophenyl
6f	methyl	3-chlorophenyl
6g	3-chlorophenyl	4-nitrophenyl
6h	4-chlorophenyl	4-nitrophenyl
6i	4-(N,N'- dimethylamino)phenyl	4-nitrophenyl
6j	4-fluorophenyl	4-nitrophenyl
6k	H	phenyl
6l	H	4-nitrophenyl
6m	H	4-chlorophenyl
6n	H	4-methoxyphenyl
6o	H	3-chlorophenyl
6p	ethyl	3-chlorophenyl

Pharmacology

Cytotoxicity Screening: MTT assay

Individual wells of a 96-well tissue culture micro titer plate were inoculated with 100 µL of complete medium containing 1×10^4 cells. The plates were incubated at 37 ^{0}C in a humidified5% CO_2 incubator for 18 hours prior to the experiment. After medium removal, 100 µL of fresh medium containing the test compounds and etoposide (Eto) at different concentrations such as 0.5, 1, and 2µM were added to each well and incubated at 37 ^{0}C for 24 hours [41-42]. Then the medium was discarded and replaced with 10 µL MTT dye. Plates were incubated at 37 ^{o}C for 2 hours. The resulting formazan crystals were solubilized in 100 µL extraction buffer. The optical density (O.D) was read at 570 nm with microplate reader (Multi-mode Varioskan Instrument-Themo Scientific). The percentage of DMSO in the medium never exceeded 0.25%.

RESULTS AND DISCUSSION

***In Silico* Studies**

As shown in Table 2, the docking scores of all the compounds were found to be in negative values and are within similar range. The negative values of the docking scores suggest that binding of the compounds in the binding site of legumain (PDB ID: 5LUB) are thermodynamically favorable.

Top scored poses of the test compounds in GLIDE XP docking are shown in Supple. Figure 1, **(supplementary data)**. The molecular interactions with the key amino acid residues in the binding site are shown as 2D interaction diagrams and their respective orientations are shown as 3D poses. The key amino acid residues in the binding site that participate in interaction with the test compounds are found to be Serine 216, Arginine 44, Histidine 45, and Tyrosine 217. The Serine 216 engages the amino group in the side chain through hydrogen bond. In majority of the compounds, van der Waals interactions are found between Arginine 44 and Histidine 45 with benzotriazole; Arginine 44 with aromatic ring at R, and Tyrosine 217 with aromatic ring at Ar. As opposed to the standard XP docking studies, Induced Fit Docking (IFD) is a program where ligands are docked into the flexible binding site. In general, the proteins in a biological environment are flexible and thus XP docking studies, in which protein is rigid, may not accurately predict the binding affinity. In IFD studies, the binding site, 5 Å around the bound ligand, is flexible and thus provide more accurate predictions on the binding affinity.

Table 2. shows the XP docking and IFD scores of the test compounds in Induced Fit Docking studies

Title	Glide XP Docking score(Kcal/mole)	IFD Score
Cpd 6a	-4.909	-596.28
Cpd 6b	-4.320	-596.08
Cpd 6c	-2.654	-599.62
Cpd 6d	-5.045	-596.44
Cpd 6e	-4.944	-598.03
Cpd 6f	-4.110	-596.37
Cpd 6g	-4.373	-596.38
Cpd 6h	-4.659	-596.50
Cpd 6i	-5.104	-597.10
Cpd 6j	-4.639	-596.59
Cpd 6k	-4.252	-596.11
Cpd 6l	-4.999	-596.90
Cpd 6m	-4.717	-597.50
Cpd 6n	-5.644	-597.63
Cpd 6o	-4.507	-596.74
Cpd 6p	-4.467	-597.27

Table 3. The total binding energy of the top scored compound/protein complex and the contributions from important interactions

Title	ΔGbind	ΔGCoulomb	ΔGCovalent	ΔGHbond	ΔGLipo	ΔGvdW
6a	-19.87	-1.43	1.69	-0.79	-13.42	-30.43
6b	-25.15	3.18	3.49	-0.49	-12.01	-30.31
6c	-55.72	8.50	1.85	-1.15	-11.65	-27.27
6c	-25.06	-8.06	0.25	-1.23	-13.24	-27.57
6d	-21.65	-2.55	2.72	-0.50	-13.26	-29.41
6e	-29.60	-4.31	2.59	-0.78	-18.67	-31.02
6f	-26.68	-4.17	2.40	-0.82	-18.24	-29.65
6g	-24.78	-5.28	2.67	-0.96	-19.43	-30.58
6h	-27.01	-2.19	4.44	-0.91	-21.36	-29.13
6i	-22.59	-3.36	5.56	-0.89	-18.66	-36.03
6j	-19.72	-3.22	3.89	-0.87	-13.41	-28.27
6k	-20.70	-3.79	3.33	-0.74	-12.44	-27.60
6l	-24.09	-1.24	3.32	-0.75	-12.50	-28.49
6m	-23.84	-4.93	3.24	-0.73	-13.33	-27.87
6n	-21.37	-4.01	3.33	-0.75	-12.62	-28.18
6o	-24.16	-3.76	1.76	-0.67	-14.24	-28.57
6p	-26.40	-3.88	3.67	-0.81	-19.58	-29.88

ADMET Studies

The drug-like properties of the test compounds are shown in Table 4. The predicted properties ofcompounds fall within the acceptable range. From this *in silico* study, none of the compounds were predicted to have CNS activity therefore, these compounds do not produce CNS effects. The octanol/water partition coefficient (QPlogPo/w) and aqueous solubility (QPlogS) of all the compounds were found to be within the acceptable range suggesting that these compounds do nothave any issues related to solubility. The compounds were also predicted to display no issue in Caco-2 cell permeability (QPPCaco) and MDCK cell permeability (QPPMDCK) as the values fall above the value of 500, indicating they have great permeability. The predicted brain/blood partition coefficient (QPlogBB) of the compounds also fall within the range, suggesting that there will be no foreseeable issue with blood-brain barrier. Similarly, the predicted values of ligand binding with serum albumin also show favourable results suggesting that the ligands will not participate in non-selective protein binding with human albumin in the circulatory system. The ligands were also predicted to show high human oral absorption based on the percent human oral absorption values. However, the predicted values for HERG toxicity (QPlogHERG) of **6a-p**

do fall outside of the recommended range, implying that these compounds may show toxic effects on heart.

Table 4. Prediction of drug like properties of the test compounds using QikProp

Title	#rotor	CNS	QPlogPo/w	QPlogS	QPlogHERG	QPPCaco	QPlogBB	QPPMDCK	#metab	QPlogKhsa	Percent Human Oral Absorption
Cpd 6a	5	0	3.11	-3.93	-5.42	2183.59	-0.39	1150.68	5	0.09	100.00
Cpd 6b	5	-2	1.52	-3.76	-5.47	32.61	-2.25	12.23	5	-0.03	62.92
Cpd 6c	3	0	2.01	-2.85	-5.36	1218.95	-0.47	612.76	4	-0.24	93.93
Cpd 6c	3	0	1.99	-2.84	-5.35	1167.86	-0.49	585.05	4	-0.24	93.48
Cpd 6d	5	0	3.51	-4.70	-5.60	2186.29	-0.43	1152.22	5	0.27	100.00
Cpd 6e	4	1	4.08	-4.69	-5.31	3196.17	0.02	4289.35	2	0.39	100.00
Cpd 6f	3	1	3.61	-4.16	-5.00	2757.51	0.02	3265.45	3	0.27	100.00
Cpd 6g	5	-1	4.30	-5.74	-6.26	408.28	-0.99	463.53	5	0.65	100.00
Cpd 6h	5	-1	4.29	-5.73	-6.25	409.74	-0.99	465.65	4	0.64	100.00
Cpd 6i	6	-2	4.24	-5.97	-6.48	339.17	-1.39	153.74	5	0.71	100.00
Cpd 6j	5	-2	4.03	-5.35	-6.20	408.78	-1.03	340.46	4	0.57	100.00
Cpd 6k	3	0	2.90	-3.43	-5.56	2170.72	-0.24	1143.35	3	0.06	100.00
Cpd 6l	4	-2	2.22	-3.60	-5.51	269.95	-1.21	120.13	3	0.02	83.43
Cpd 6m	3	0	3.39	-4.18	-5.52	2170.95	-0.08	2823.53	2	0.17	100.00
Cpd 6n	4	0	3.00	-3.68	-5.48	2165.94	-0.32	1140.63	3	0.08	100.00
Cpd 6o	3	0	3.39	-4.18	-5.52	2170.94	-0.08	2818.46	3	0.17	100.00
Cpd 6p	4	1	4.03	-4.53	-5.20	3269.61	0.02	3928.24	3	0.38	100.00

Note: #rotor, Number of non-trivial (not CX3), non-hindered (not alkene, amide, small ring) rotatable bonds: recommended range 0-15; CNS, predicted central nervous system activity: –2 (inactive) to +2 (active) scale; QPlogPo/w, Predicted octanol/water partition coefficient: recommended value -2.0 – 6.5; QPlogS, Predicted aqueous solubility, log S. S in mol dm–3 is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid: recommended range -6.5 – 0.5; PlogHERG, Predicted IC50 value for blockage of HERG K+ channels: recommended value below - 5; QPPCaco, Predicted apparent Caco-2 cell permeability in nm/sec. Caco2 cells are a model for the gut-blood barrier: <25 = poor, >500 = great; QPlogBB, Predicted brain/blood partition coefficient: recommended range -3.0 – 1.2; QPPMDCK Predicted apparent MDCK cell permeability in nm/sec: <25 = poor, >500 = great; QPlogKhsa, Prediction of binding to human serum albumin: recommended range -1.5 – 1.5; Percent Human Oral Absorption, Predicted human oral absorption on 0 to 100% scale: > 80% = high, <25% = poor

Chemistry

Synthesis of new Mannich base derivatives of benzotriazoles (**6a-p**) was depicted in Scheme 1. The commercial available compound benzotriazole (**3**) undergoes to Mannich reaction with aliphatic and aromatic aldehydes (**4a-g**) and different types of aryl amines (**5a-i**) in ethanol at room temperature for 1 hour to give pure final compounds **6a-p.** Further, these compounds were characterized by ^{1}HNMR, ^{13}CNMR and mass spectral data. The proton values of compound **6a** was appeared at 3.89 ppm as singlet (s,(–OCH_3)2),the methylene proton appear at 4.14 ppm as a singlet (s, –CH_2-), and the amine proton showed at 6.57 ppm (t, –NH-), as well as the ^{13}CNMR value of –CH_2- peak appear at 57.6 ppm. The mass value of compound m/z: 285 $[M+H]^+$.

Spectral Characterization

N-((1H-Benzo[d][1,2,3] triazol-1-yl)methyl)-3,5-dimethoxybenzenamine (6a)

^{1}H NMR (400 MHz, DMSO-d6): □ 3.92 (s, 6H), 6.10 (d, 2H, *J* = 6.2 Hz), 6.83 (s, 1H), 7.20 (s, 2H), 6.90 (t, 1H), 7.35 (t, 1H), 7.49 (dd, 1H, *J* = 7.01, *J* = 8.0 Hz), 7.98 (d, 1H, *J* = 8.22 Hz), 8.08 (d, 1H, *J* = 8.22 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 57.6, 59.4, 104.6, 108.4, 112.8, 122.7, 126.2, 129.7, 134.5, 142.7, 147.6, 163.4; MS (ESI): m/z 285 $[M+H]^+$.

N-((1H-benzo[d][1,2,3] triazol-1-yl)methyl)-3,5-dinitrobenzenamine (6b)

^{1}H NMR 6.17 (d, 2H, J = 7.6 Hz), 6.96 (t, 1H), 7.43 (t, 1H), 7.62 (dd, 1H, *J* = 7.4, J = 8.3 Hz), 7.92 (s, 2H), 8.04 (d, 1H, *J* = 8.3 Hz), 8.10 (d, 1H, *J* = 8.3 Hz), 8.75 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d6): □ 58.5, 105.4, 106.6, 112.8, 121.4, 126.7, 129.7, 134.5, 142.5, 143.6, 147.6; MS (ESI):m/z 315 $[M+H]^+$.

N-((1H-benzo[d][1,2,3] triazol-1-yl)methyl)pyridin-4-amine (6c)

^{1}H NMR (400 MHz, DMSO-d6): □ 6.21 (d,2H, *J* = 7.68 Hz), 6.97 (t, 1H), 7.44 (t, 1H), 7.55 (d, 2H, J = 7.34 Hz), 7.62 (dd, 1H, *J* = 7.5, *J* = 8.3 Hz), 8.10 (d, 1H, *J* = 8.5 Hz), 8.13 (d, 1H, *J* = 8.5 Hz), 8.45 (d, 2H, *J* = 7.34 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 59.5, 112.8, 114.6, 121.7, 126.5, 129.3, 135.5, 142.7, 147.3, 151.6; MS (ESI): m/z 226 $[M+H]^+$.

N-(1-(1H-Benzo[d][1,2,3]triazol-1-yl)propyl)-4-chlorobenzenamine (6e)

^{1}H NMR (400 MHz, DMSO-d6):□ 0.96 (t, 3H), 1.89-2.01

(m, 2H), 6.10-6.12 (m, 1H), 6.89 (d, 2H, *J* = 8.8 Hz), 7.14 (d, 2H, *J* = 8.8 Hz), 6.95 (t, 1H), 7.36 (t, 1H), 7.58 (dd, 1H, *J* = 7.4, *J* = 8.2 Hz), 8.05 (d, 1H, *J* = 8.6 Hz), 8.09 (d, 1H, *J* = 8.6 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 10.2, 28.4, 82.4, 112.3, 118.5, 119.9, 120.8, 121.2, 126.3, 129.3, 134.4, 142.4, 143.7, 147.6; MS (ESI): m/z 287 $[M+H]^+$.

N-(1-(1H-Benzo[d][1,2,3]triazol-1-yl)ethyl)-3-chlorobenzenamine (6f)

^{1}H NMR (400 MHz, DMSO-d6): □ 1.62 (d, 3H, *J* = 6.9 Hz), 6.08-6.11 (m, 1H), 6.97 (t, 1H), 7.22 (d, 1H, *J* = 8.1 Hz), 7.28 (t, 1H), 7.38 (t, 1H), 7.43 (d, 1H, *J* = 8.6 Hz), 7.48 (s, 1H), 7.57 (dd, 1H, J= 7.4, *J* = 8.2 Hz), 8.06 (d, 1H, *J* = 8.9 Hz), 8.10 (d, 1H, *J* = 8.9 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 22.7, 82.4, 112.3, 119.9, 120.5, 121.2, 126.7, 129.3, 134.4, 142.7, 143.4, 147.6; MS (ESI):m/z 273$[M+H]^+$

N-((1H-Benzo[d][1,2,3]triazol-1-yl)(3-chlorophenyl)methyl)-4-nitrobenzenamine (6g)

^{1}H NMR (400 MHz, DMSO-d6): □ 6.17 (d, 2H, *J* = 7.7 Hz), 6.98 (t, 1H), 7.31 (d, 1H, J = 8.3 Hz), 7.35-7.39 (m, 2H), 7.47 (d, 1H, *J* = 8.5 Hz), 7.55-7.61 (m, 2H), 7.86 (d, 2H, *J* = 7.7 Hz), 7.93 (d, 2H, *J* = 7.7 Hz), 8.10 (d, 1H, *J* = 8.8 Hz), 8.14 (d, 1H, *J* = 8.8 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 82.6, 112.6, 119.7, 120.6, 122.4, 126.3, 128.4, 129.4, 130.7,131.8, 132.7, 133.5, 134.5, 139.5, 142.7, 143.2, 147.1;MS (ESI): m/z 380 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)(4-chlorophenyl)methyl)-4-nitrobenzenamine (6h)

^{1}H NMR (400 MHz, DMSO-d6): □ 6.18 (d, 2H, *J* = 7.8 Hz), 6.98 (t, 1H), 7.48 (d, 2H, *J* = 8.2 Hz), 7.38 (t, 1H), 7.51 (d, 2H, *J* = 8.2 Hz), 7.62 (dd, 1H, *J* = 7.8, J = 8.4 Hz), 7.87 (d, 2H, *J* = 7.6 Hz), 7.94 (d, 2H, *J* = 7.6 Hz), 8.11 (d, 1H, *J* = 8.9 Hz), 8.13 (d, 1H, *J* = 8.9 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 82.6, 112.4, 119.5, 120.7, 122.4, 126.5, 130.4, 131.4, 132.6, 134.2, 138.3, 139.6, 142.2, 142.3,147.3; MS (ESI): m/z 380 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)(4-(dimethylamino)phenyl)methyl)-4-nitrobenzenamine (6i)

^{1}H NMR (400 MHz, DMSO-d6): □ 2.83 (s, 6H), 6.15 (d, 2H, *J* = 7.4 Hz), 6.96 (t, 1H), 7.43 (d, 2H, *J* = 8.0 Hz), 7.37 (t, 1H), 7.50 (d, 2H, *J* = 8.0 Hz), 7.58 (dd, 1H, *J* = 7.3, *J* = 8.2 Hz), 7.86 (d, 2H, *J* = 7.7 Hz), 7.93 (d, 2H, *J* = 7.7 Hz), 8.09 (d, 1H, *J* = 8.8 Hz); ^{13}C NMR (75 MHz, DMSO- d6): □ 42.3, 82.6, 112.4, 115.6, 119.4,120.4,122.6, 126.5, 129.7,130.5,134.2, 139.6, 142.3,142.6, 147.2,153.4; MS (ESI): m/z 389 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)(4-fluorophenyl)methyl)-4-nitrobenzenamine (6j)

^{1}H NMR (400 MHz, DMSO-d6): □ 6.16 (d, 2H, *J* = 7.3 Hz), 6.94 (t, 1H), 7.45 (d, 2H, J = 8.1 Hz), 7.38 (t, 1H), 7.51 (d, 2H, *J* = 8.1 Hz), 7.58 (dd, 1H, *J* = 7.3, *J* = 8.2 Hz), 7.87 (d, 2H, J = 7.8 Hz), 7.94 (d, 2H, J = 7.8 Hz), 8.07 (d, 1H, J = 8.8 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 82.4, 112.5, 117.4, 120.7, 122.4, 126.5, 127.6,129.7, 130.5, 134.2, 139.3, 142.4,148.6,154.7, 165.5; MS (ESI): m/z 364 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)methyl)benzenamine (6k)

^{1}H NMR (400 MHz, DMSO-d6) δ: 8.04 (d, *J* = 8.4 Hz, 1H), 7.95 (d, *J* = 8.4 Hz, 1H), 7.51 (t, *J* = 7.4 Hz, 1H), 7.35 (t, *J* = 7.4 Hz, 1H), 7.27 (t, *J* ＝7.0 Hz, 1H), 7.09 (t, *J* ＝7.5 Hz, 2H), 6.84 (d, *J* ＝8.1 Hz, 2H), 6.57 (t, *J* =7.2 Hz, 1H), 6.11 (d, *J* = 7.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d6): □ MS (ESI): m/z 225 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)methyl)-4-nitrobenzenamine (6l)

^{1}H NMR (400 MHz, DMSO-d6) δ: 8.47 (t, *J* = 6.6 Hz, 1H), 8.08-8.04 (m, 4H), 7.62-7.56 (m, 1H), 7.47－7.42 (m, 1H), 6.96 (d, *J* = 8.0 Hz, 1H), 6.25 (d, *J* = 6.8 Hz, 1H), 6.10 (d, *J* = 7.1 Hz, 2H);

^{13}C NMR (75 MHz, DMSO-d6): □ 59.4, 112.4, 119.5, 120.5, 120.8, 121.7, 126.6, 129.5, 134.3, 142.5, 143.7, 147.8; MS (ESI): m/z 270 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)methyl)-4-chlorobenzenamine (6m)

^{1}H NMR (400 MHz, DMSO-d 6) δ 8.07-8.03 (m, 2H), 7.57 (t, *J* = 7.4 Hz, 1H), 7.43 (t, *J* = 7.4 Hz, 1H), 7.36 (t, *J* = 7.6 Hz, 1H), 7.14 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 2H), 6.11 (d, *J* = 7.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d6): □ 59.6, 112.4, 122.6, 123.9, 126.5, 129.6, 131.3, 132.2, 134.5, 142.7, 147.4; MS (ESI): m/z 259 $[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)methyl)-4-methoxybenzenamine (6n)

^{1}H NMR (400 MHz, DMSO-d6) δ: 8.03 (d, *J* = 8.2 Hz, 1H), 7.95 (d, *J* = 8.2 Hz, 1H), 7.51 (dd, *J* =7.2 8.0 Hz 1H), 7.36 (t, *J* = 7.5 Hz, 1H), 6.91 (t, *J* = 7.5 Hz, 1H), 6.76 (d, *J* = 8.6 Hz, 2H), 6.64 (d, *J* = 8.6 Hz, 2H), 6.03 (d, *J* = 6.2 Hz, 2H), 3.59 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d6): □ 57.6, 59.4, 112.8, 116.4, 122.3, 123.7, 126.6, 129.7, 134.5, 142.4, 147.6, 158.3; MS (ESI): m/z 255$[M+H]^+$.

N-((1H-Benzo[d][1,2,3]triazol-1-yl)methyl)-3-chlorobenzenamine (6o)

^{1}H NMR (400 MHz, DMSO-d6): □ 6.14 (d, 2H,), 6.61–6.64 (d, 1H, *J* = 8.0 Hz), 6.77–6.79 (d, 1H, *J* = 7.9 Hz), 6.88–6.89 (m, 1H), 7.11 (s, 1H), 7.37–7.41 (m, 1H), 7.56 (d, 1H, *J* = 8.3 Hz), 7.76-7.84 (m, 2H), 8.04 (d, 1H, *J* = 8.3 Hz); ^{13}C NMR (100 MHz, DMSO-d6): □ 56.3, 111.0, 111.5, 112.2, 117.2, 119.0, 124.0, 127.2,130.5, 132.0, 133.6, 145.3, 147.3; MS (ESI): m/z 259 $[M+H]^+$.

N-(1-(1H-Benzo[d][1,2,3]triazol-1-yl)propyl)-3-chlorobenzenamine (6p)

^{1}H NMR (400 MHz, DMSO-d6):□ 0.96 (t, 3H), 1.91-2.04 (m, 2H), 6.10-6.12 (m, 1H), 6.71 (d, 1H, *J* = 8.2 Hz), 6.83 (d, 1H, *J* = 8.2 Hz), 7.05 (s, 1H), 7.26 (t, 1H), 7.45-7.57 (m, 3H), 8.09 (d, 1H, *J* = 8.6 Hz); ^{13}C NMR (75 MHz, DMSO-d6): □ 10.3, 27.8, 62.6, 109.1, 117.6, 119.0, 120.2, 120.6, 124.7, 126.8, 128.7, 132.0, 134.6, 141.5, 144.3, MS (ESI): m/z 287 $[M+H]^+$.

Pharmacology

Cytotoxicity Screening: MTT assay

These compounds (**6a-p**) were investigated for their anticancer properties towards four different human cancer cell lines including MCF-7 (breast cancer), A549 (lung cancer), Colo-205 (colon cancer) and A2780 (ovarian cancer) by utilize of MTT method. The obtained results were summarized in Table 5, the clinical drug as etoposide used as positive control. Most of the compounds were displayed good to moderate anticancer activities to compare with control drug. The compounds showed IC_{50} values range from 0.012±0.001 to 22.9±9.11 µM, and positive control showed values from 0.13 ± 0.017 to 3.08 ± 0.135µM. Among synthesized compounds, these **6a, 6c, 6e, 6f**, **6j**, **6n** and **6p** were demonstrated more potent anticancer activities than etoposide. Further, all compounds were examined for structure-activity relationship study; those results indicated that the compound **6a** contain electron-donating (3,5-dimethoxy) group on the phenyl ring displayed highest anticancer activity on four cancer cell lines with IC_{50} values as MCF- 7=0.012±0.001µM, A549=0.18±0.076µM, Colo-205=0.34±0.083µM and A2780= 0.07±0.006µM, respectively. Compound **6n** contain 4-methoxy substituent on the aryl skeleton showed somewhat lower activity (MCF-7=0.97±0.062µM, A549= 1.87±0.51µM, Colo-205= 2.06±0.33µM and A2780= 2.33±1.64µM) to compare with **6a.** The replacement of phenyl with 4- pyridyl ring resulted compound **6c** was showed good activity on all cell lines (MCF- 7=1.65±0.18µM, A549=0.72±0.044µM, Colo-205=1.27±0.39µM and A2780= 2.08±1.82µM).

The compound **6b** contain 3,5-dinitro group on the phenyl ring was displayed very poor activity. Whereas, compound **6e** with 4-chloro substitution on the aryl ring and ethyl functionality was showed good activity on four cell lines (MCF-7=1.98±0.60µM, A549=0.79±0.029µM, Colo-205=2.12±1.76µM and A2780=1.84±0.63µM). As well as compound **6f** with electron- withdrawing group (3-chloro) present at 3rd position of the phenyl moiety and methyl group was showed improved activity (MCF-7=1.02±0.34µM, A549=1.18±0.45µM, Colo-205=0.57±0.068µM and A2780= 0.19±0.55µM) to compare with **6e.** Similarly, compound **6p** with electron withdrawing group (3-chloro), (R = ethyl) was displayed loss of activity (MCF-7= 1.59±0.73µM, A549= 2.44±1.84µM, Colo-205= 1.88±0.63µM and A2780= 2.27±1.49µM) on particularly two cell lines (A549, A2780) than **6e** and **6f.** Interestingly compound **6j** with 4-nitrogroup on the phenyl ring and 4-fluorophenyl on the methylene carbon was second highest anticancer activity (MCF-7=0.076±0.002µM, A549=0.23±0.045µM, Colo-205=0.64±0.031µM and A2780= 0.75±0.063µM). Compounds **6g** (Ar = 4-nitrophenyl and R = 3-chlorophenyl), **6h** (Ar = 3-chlorophenyl and R = 4-chlorophenyl), **6i** (AR = 4-chloro and R = 4-N, N'-dimethylamino**), 6l (**Ar = 4-nitrophenyl and R = H), **6m (**Ar = 4-chlorophenyl and R = H), and **6o (**Ar = 3-chlorophenyl and R = H) skeletons attached to the methylene carbon were showed acceptable activities.

Table 5. In vitro cytotoxicity of newly compounds 6a-p with IC50 in µM.

Compound	[c]MCF-7	[d]A549	[e]Colo-205	[f]A2780
6a	0.012±0.001	0.18±0.076	0.34±0.083	0.07±0.006
6b	-	16.4±5.88	7.89±6.78	13.5±4.18
6c	1.65±0.18	0.72±0.044	1.27±0.39	2.08±1.82
6e	1.98±0.60	0.79±0.029	2.12±1.76	1.84±0.63
6f	1.02±0.34	1.18±0.45	0.57±0.068	0.19±0.55
6g	3.98±2.48	7.12±5.87	12.6±7.54	9.34±6.88
6h	3.77±2.36	3.28±2.68	-	10.5±1.67
6i	2.77±1.57	-	3.19±1.98	3.22±2.12
6j	0.076±0.002	0.23±0.045	0.64±0.031	0.75±0.063
6k	18.5±6.43	22.9±9.11	3.90±4.92	6.27±3.76
6l	4.85±2.71	7.53±3.68	3.88±2.17	-
6m	9.56±4.53	-	-	15.7±6.46
6n	0.97±0.062	1.87±0.51	2.06±0.33	2.33±1.64
6o	3.47±1.97	8.55±4.32	-	-
6p	1.59±0.73	2.44±1.84	1.88±0.63	2.27±1.49
Etoposide	2.11 ± 0.024	3.08 ± 0.135	0.13 ± 0.017	1.31 ± 0.27

"-" = Not active.

[a]Each data represents as mean ±S. D values. From three different experiments performed in triplicates. [bc]MCF-7: human breast cancer cell line. [d]A549: human lung cancer cell line. [e]Colo- 205: human colon cancer cell line. [f]A2780: human ovarian cancer cell line.

CONCLUSION

The molecular interactions with the key amino acid residues in the binding site that participate ininteraction with the test compounds are the same as those found in XP docking. Thus, the IFD results are in agreement with those of XP docking studies, confirming the binding of the test compounds in the binding site of legumain. The predicted ADMET properties of compounds fallwithin the acceptable range. From this *in silico* study, none of the compounds were predicted to have CNS activity therefore, these compounds do not produce CNS effects. New mannich base derivatives of benzotriazoles (**6a-p**) were synthesized

and these compounds were characterized by ^{1}HNMR, ^{13}CNMR and mass spectral data. These compounds (**6a-p**) were investigated for their anticancer properties towards four different human cancer cell lines by utilize of MTT method. the clinical drug as etoposide used as positive control. Most of the compounds were displayed good to moderate anticancer activities to compare with control drug. The compounds showed IC_{50} values range from 0.012±0.001 to 22.9±9.11 µM, and positive control showed values from 0.13 ± 0.017 to 3.08 ± 0.135µM. Among synthesized compounds, these **6a, 6c, 6e, 6f**, **6j**, **6n** and **6p** weredemonstrated more potent anticancer activities than etoposide. Further, all compounds wereexamined for structure-activity relationship study; those results indicated that the compound **6a**contain electron-donating (3,5-dimethoxy) group on the phenyl ring displayed highest anticancer activity on four cancer cell lines with IC_{50} values as MCF-7=0.012±0.001µM, A549=0.18±0.076µM, Colo-205=0.34±0.083µM and A2780= 0.07±0.006µM, respectively.

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