EATON'S REAGENT CATALYZED SYNTHESIS, IN VITRO α-AMYLASE INHIBITORY ACTIVITY AND MOLECULAR DOCKING STUDY OF SOME SCHIFF'S BASES AS DIABETIC-II INHIBITORS

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Keywords: *α*-Amylase, Eaton's reagent, Schiff's bases, Solvent-free, Molecular docking

A series of Schiff's bases of p-tolylsulphonyl hydrazide were synthesized by using Eaton's reagent under solvent-free condition, characterized by spectroscopic data and for evaluated α-amylase inhibitory activity *in vitro*. Four among the studied compounds exhibited varying degrees of α-amylase inhibitory activity with IC_{50} values in the range of 115.48 to 169.42 μg mL^{-1}. The observed results were supported by the molecular docking study performed to understand the binding interaction of the title compounds with the active site of α-amylase enzyme. Results suggest that Schiff's bases of p-tolylsulphonyl hydrazide derivatives can act as potential antidiabetic drugs.

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INTRODUCTION

Diabetes mellitus, a metabolic disorder characterized by chronic hyperglycemia condition, has become a significantly growing disease all over the world due to changing lifestyles and increasing junk food in the diet. As per the reports of the International Diabetes Federation (IDF), approximately 366 million people have diabetes and the number may be doubled by 2030.[1] It is mainly caused by the lack of insulin, which includes increased morbidity, disability, mortality and represents a threat to the economies of all countries, especially in developing ones like India.[2] In diabetic patients, the immune system becomes weak by destroying insulin-producing cells, leading to a high level of blood glucose, which significantly increases the risk of long-term heart disease, stroke, dysfunction and kidney failure.[3]

α-Amylase is an enzyme that catalyzes the hydrolysis of carbohydrates and starch into glucose in human blood, resulting in hyperglycemic condition and eventually type-II Diabetes mellitus (T2DM). It is present in the saliva of human beings, where it begins the chemical process of digestion. Pancreas and salivary glands make α-amylase to hydrolyze dietary starch into disaccharides which are then converted by other enzymes to glucose that acts as a source of energy to the body. Inhibitors of α-amylase function by modulating the blood glucose level after a meal.[4] The ability of a drug to delay the production and absorption of glucose by inhibiting carbohydrate hydrolyzing enzymes - α-amylase and α-glucosidase is an important therapeutic approach for the development of antidiabetic drugs. Thus α-amylase functioning as a key enzyme for the digestion and absorption of starch in the blood is one of the targets in the treatment of T2DM.

Schiff's bases are the condensation products of primary amines with carbonyl compounds, named after the scientist Hugo Schiff. Schiff's bases possess imine or azomethine (–C=N–) functional group and constitute a versatile pharmacophore and reaction intermediates for the design, synthesis, and development of various bioactive lead compounds of various biological interest such as 4-thiazolidinone,[5] inorganic metal complexes[6] and azetidinones.[7] Furthermore, they are also well known to exhibit numerous biological activities such as antimicrobial,[8] antitubercular,[9] antioxidant,[10] anticonvulsant,[11] anti-inflammatory,[12] and anticancer activity.[13] They are also useful in pigments and dyes,[14] intermediates for organic synthesis,[15] polymer stabilizers[16] and corrosion inhibitors.[17]

Sulfonamide functionality is the basis of several drugs. Sulfamethoxazole (**a**) is an antibacterial sulfa drug. Acetohexamide (**b**) is a sulfonylurea used to treat diabetes mellitus. Ethoxzolamide (**c**) is a sulfonamide drug that acts on carbonic anhydrase inhibitors and widely used in the treatment of glaucoma and ulcers.[18] Mafenide (**d**), also known as sulfamylon, is a sulfonamide type medication that is used as an antibiotic. Furosemide (**e**) is used to treat fluid build-up due to heart failure, liver scarring or kidney disease and also in the treatment of high blood pressure.

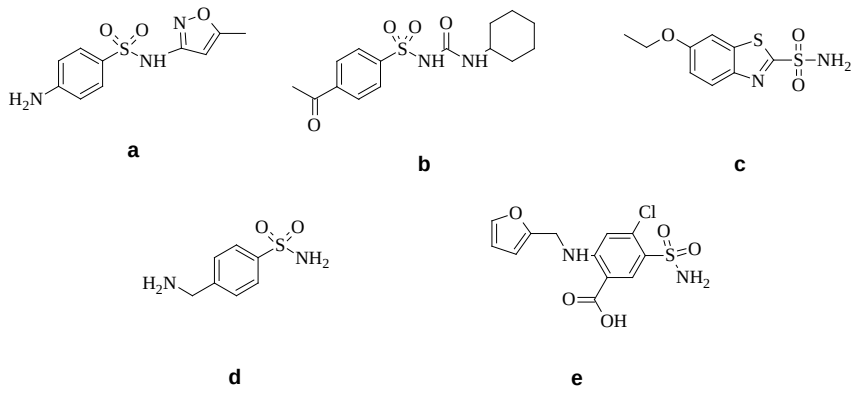
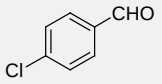
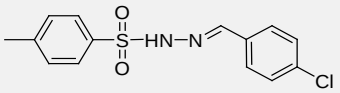
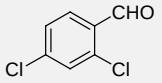
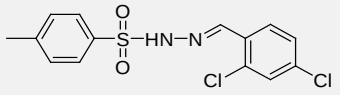
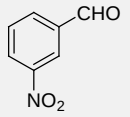
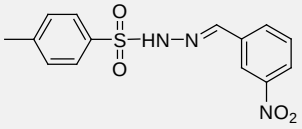
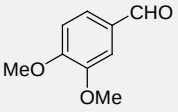
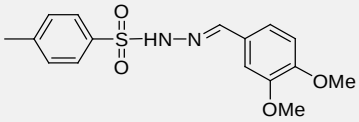
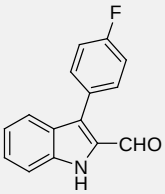
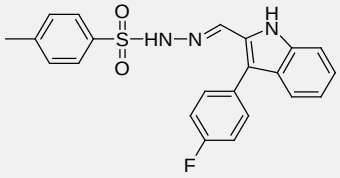
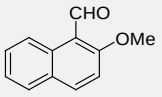
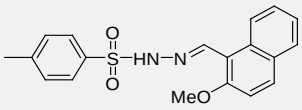
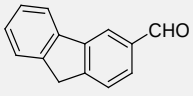
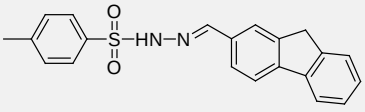


Figure 1: Some biologically active sulphonamide drugs

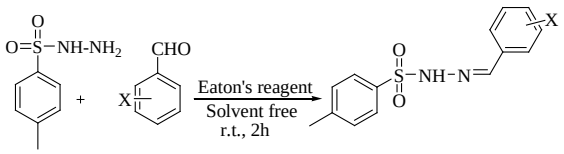
Table 1. Yields of Eaton's reagent catalyzed synthesis of Schiff's bases

Entry	Aldehyde	Schiff's base	Code	Yield*, %	M. P., ºC
1			**1**	91	133
2			**2**	90	178-180
3			**3**	89	142-143
4			**4**	90	134-136
5			**5**	80	133-135
6			**6**	90	182-183
7			**7**	92	177-179

*Yields isolated in case of p-toluenesulphonyl hydrazide (5 mmol), aldehyde (5 mmol) using Eaton's reagent (5 mol %) under solvent-free conditions.

Eaton's reagent [P_2O_5: methane sulphonic acid] (1:10 by weight) is used as an alternative to the polyphosphoric acid (PPA) in organic synthesis.[19] It is inexpensive, easy to handle, easy to separate, less viscous than PPA and commercially available reagent. Philip Eaton firstly synthesized it in 1973. Eaton's reagent has been used for the synthesis of phenanthrolines,[20] tetrahydroisoquinoline-2-ones,[21] quinolones,[22] 3,4-dihydropyrimidin-2(1H)-ones,[23] polyamidines[24] and chalcones.[25] Thus, considering the widespread applications, biological significance of Schiff's bases in various fields and in continuation of our successful attempts in the development of bioactive heterocyclic

compounds,[26-27] in the present article we report the synthesis of some Schiff's bases from p-tolyl sulphonyl hydrazide and various aldehydes using Eaton's reagent under solvent-free conditions (Scheme 1).



Scheme 1. Eaton's reagent catalyzed synthesis of Schiff's bases.

The synthesized compounds were screened for *in vitro* α-amylase enzyme inhibitory antidiabetic activity whose observed results were supported by computational studies of molecular docking in terms of possible interactions with the active site of the α-amylase enzyme and high free energy of binding.

RESULTS AND DISCUSSION

In the present work, we synthesized some Schiff's bases of p-toluenesulphonyl hydrazide with various aldehydes under solvent-free conditions at room temperature. p-Toluenesulphonyl hydrazide is a solid compound. After the addition of aldehyde to p-toluenesulphonyl hydrazide, initially, the reaction mixture was difficult to stir at room temperature, particularly in the case of solid aldehydes. But as soon as Eaton's reagent was added to the above mixture, an exotherm was observed, the contents turned into the liquid phase for a while and then a white or yellow solid mass appeared which was stirred at room temperature for 2 h.

Initially, we tried the reaction of benzaldehyde with p-toluene sulphonyl hydrazide using Eaton's reagent (5 mol%) under solvent-free conditions. When tried with different amounts of Eaton's reagent from 5 to 10, 15 and 20 mol %, 5 mol % of the reagent was found to be appropriate for this transformation. Commonly ethanol is the best solvent for the synthesis of Schiff's bases. However, the use of ethanol in this reaction required slightly more time (3 h) for completion. Thus the use of 5 mol% of Eaton's reagent under solvent-free conditions was found to be the best condition for this reaction. Although the synthesis of Schiff's bases is fast and straightforward, comparatively more time was required for completion of the reaction (2 h) which could be most probably due to the presence of a sulphonamide group adjacent to the reaction center - NH_2.

Under these reaction conditions, various aldehydes were treated with p-toluene sulphonyl hydrazide to afford the corresponding Schiff's bases in excellent yields in almost all cases. Electron withdrawing groups reacted more smoothly, providing higher yields as compared to those with electron-donating groups. To our delight, methoxy groups in the Schiff's bases remained intact under these reaction conditions (Table 1).

The synthesized compounds were evaluated for amylase inhibitory activity and the results were compared with the standard acarbose. Among all the synthesized Schiff's bases, compounds **1** (4-chlorophenyl), **3** (3-nitrophenyl), **5** (fluorophenyl substituted indole) and **6** (2-methoxynaphthyl ring) had significant amylase inhibitory activity, but less than the standard acarbose at all the concentrations i.e. 20, 40, 60, 80 and 100 µg mL^{-1} (Table 2).

Molecular docking study of human pancreatic α-amylase:

α-Amylase is an enzyme that catalyzes the hydrolysis of α-1,4 glucan linkages in starch. In human beings, α-amylase is composed of 496 amino acids in a single polypeptide chain and occurs as different isozymes synthesized in either salivary glands or the pancreas.

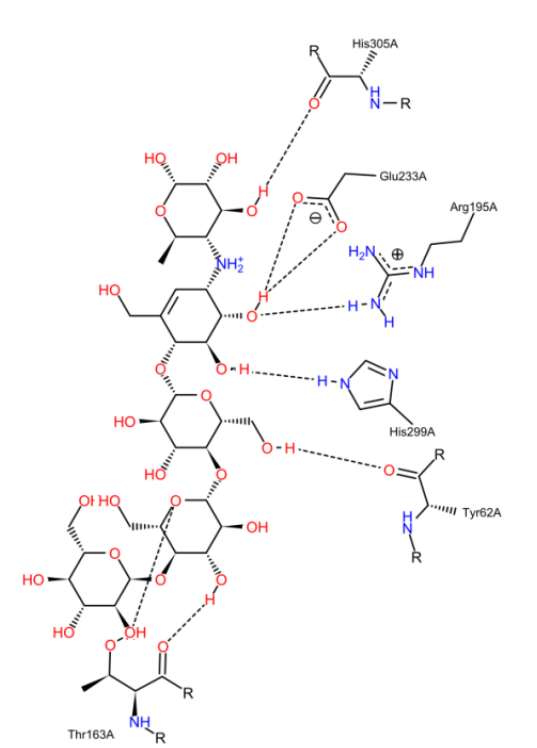


Figure 2. Interactions of acarbose with key residues at the binding site.

The screening of synthesized Schiff's bases showed that the compounds **1**, **2**, **3**, **5** and **6**possesgood α-amylase inhibitory activity with IC50 values less than 200 µM. The remaining compounds with IC_{50} higher than 200 µM possess moderate α-amylase inhibitory activity. Docking results were found in good agreement with the experimentally observed α-amylase inhibitory activity. For the α-amylase inhibitory activity, the hydrogen bond interactions with residues HIS305, GLU233, ARG195, HIS299, TYR62, and THR163 were observed to be important as shown by α-amylase inhibitor Acarbose (Figure 2).

Table 2. % Results of amylase inhibitory activity and molecular docking study

Entry	Compound	% Inhibition at concentration, µg mL^{-1}					IC_{50}, µg mL^{-1}	Free Energy of binding, kcal mol^{-1}
		20	40	60	80	100		
1	**1**	5.56	11.83	23.07	34.43	39.17	115.48	-8.06
2	**2**	19.76	23.66	27.57	35.97	14.79	190.69	-8.58
3	**3**	22.60	26.62	34.79	35.85	40.71	169.42	-8.72
4	**4**	10.76	15.73	13.37	22.13	29.23	226.22	-7.29
5	**5**	31.36	39.40	45.79	51.47	52.89	154.17	-7.79
6	**6**	45.79	50.88	60.35	63.07	69.70	126.70	-8.82
7	**7**	33.96	39.40	43.43	46.50	47.92	253.97	-7.14
8	**Acarbose**	35.14	50.88	62.13	69.94	76.09	70.656	-12.52

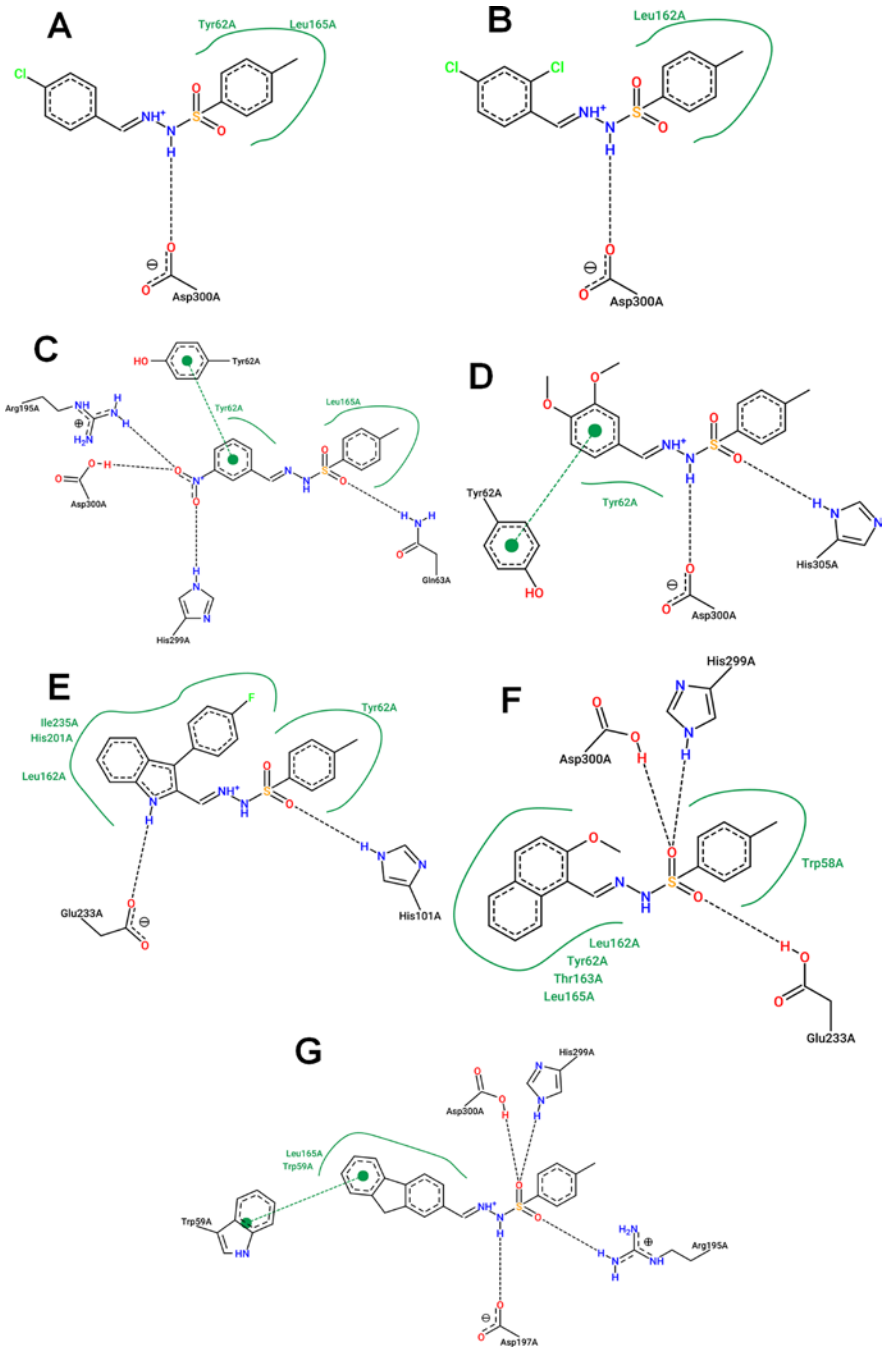


Figure 3. 2D-Interaction diagrams for all compounds. A) Compound **1**; B) Compound **2**; C) Compound **3**; D) Compound **4**; E) Compound **5;** F) Compound **6**; and G) Compound **7** .

Most of the synthesized Schiff's bases showed similar hydrogen bond interactions and the aromatic rings of these Schiff's bases were found to occupy the binding pockets at the active site of α-amylase. Aromatic rings attached to imino nitrogen in compounds **1**, **6**, **5**, **3**, and **2** were found to make crucial hydrophobic interactions with TYR62, whereas the 4-methylphenyl ring in compound **5** and **3** showed significant hydrophobic interactions with the residues HIS201, LEU162 and ILE235 (Figure 3).

For **2**, **3** and **6** Schiff's bases, hydrogen bonding was observed with residues like ASP197, GLU233, HIS299, ASP300, THR163 as the key interactions similar in the case of standard acarbose. For Schiff's bases **4** and **7**, the crucial hydrophobic interaction with TYR62 were not found, but instead, the aromatic rings attached to imino nitrogen were found to occupy the pocket surrounded by the residues TRP59, TRP58 (Figure 3). These compounds form hydrogen bonds with the residues ASP300, HIS299, ASP197, and GLU233. The crucial hydrophobic interaction at the pocket with TYR62 residues was not found in these compounds which may be responsible for their less α-amylase inhibitory activity. This suggests that the presence of naphthyl, 2-indolyl, m-nitrophenyl and ortho-substituted rings were favorable for α-amylase inhibitory activity. The electronegative chloro and nitro groups on the phenyl ring were found to be beneficial for α-amylase inhibitory activity. The aromatic rings in these compounds were found to burry in the pockets made of residues like TYR62, LEU165.

EXPERIMENTAL

Chemicals used were SD fine or Aldrich made and used without further purification. The reaction progress was monitored by using silica gel pre-coated on aluminum TLC plates using 40 % ethyl acetate in n-hexane. Melting points of the products were measured in capillaries open at one end and were uncorrected. The products were purified by recrystallization from ethanol and confirmed by analysis of spectroscopic data(^{1}H NMR, ^{13}C NMR, IR and mass spectra).

General procedure for the solvent-free synthesis of Schiff's bases using Eaton's reagent

A mixture of p-toluenesulfonyl hydrazide (5 mmol) and aldehyde (5 mmol) was stirred with Eaton's reagent (5 mol %) at room temperature under the solvent-free condition for 2 h. Progress of the reaction was monitored by TLC in 40 % ethyl acetate in n-hexane. After completion of the reaction as confirmed by TLC, the reaction mass was poured into ice-cold water, resulting in solid was separated by filtration under vacuum and further purified by recrystallization from ethanol as the pure Schiff's base.

1-(4-Chlorobenzylidene)-2-tosylhydrazine (1)

^{1}HNMR (400 MHz, $CDCl_3$): δ ppm 2.39 (s, 3H), 7.24-7.31 (dd, 4H), 7.47 (d, 2H), 7.7 (s, 1H), 7.86 (d, 2H), 8.35 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ ppm 78.65, 127.12, 127.74, 128.55, 129.34, 132.43, 134.63, 136.11, 143.16, 145.27; Mass 309.1 $(M+1)^+$.

1-(2,4-Dichlorobenzylidene)-2-tosylhydrazine (2)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.36 (s, 3H), 7.4-7.46 (m, 3H), 7.66 (s,1H), 7.7 -7.76 (dd, 3H), 8.19 (s, 1H), 11.82 (s, 1H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ ppm: 78.77, 127.11, 127.56, 129.04, 129.84, 133.49, 135.09, 136.00, 141.31, 143.36; Mass: 343.1 $(M+1)^+$.

1-(3-Nitrobenzylidene)-2-tosylhydrazine (3)

^{1}H NMR (400 HMz, $CDCl_3$) δ ppm 2.43 (s, 3H), 7.27-7.37 (dd, 2H), 7.55-7.59 (dd, 1H), 7.84-7.95 (m, 4H), 8.19 (d, 1H), 8.37(s, 1H), 8.49 (d, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ ppm: 62.99, 120.96, 122.84, 124.19, 127.63, 129.68, 132.57, 135.41, 136.01, 138.08, 144.50, 148.04; Mass 320.0 $(M+1)^+$.

1-(3,4-Dimethoxybenzylidene)-2-tosylhydrazine (4)

^{1}H NMR (400 MHz) δ ppm: 2.38 (s, 3H), 3.8 (s, 6H), 6.98 (s, 1H), 7.7 (dd, 2H), 7.4 (s, 2H), 7.8 (dd, 3H), 11.2 (s, 1H); ^{13}C NMR (100.6 MHz) δ ppm: 20.94, 55.50, 108.57, 111.45, 120.91, 126.39, 127.25, 129.55, 136.12, 143.14, 147.18, 148.96, 150.59; IR (neat) cm^{-1}: 3196, 2970, 2930, 1598, 1550, 1029; Mass: 335.2 $(M+1)^+$.

1-[3-(4-Fluorophenyl)-1H-indol-2-yl)methylene)]-2-tosylhydrazine (5)

^{1}H NMR (400 MHz) δ ppm: 2.43 (s, 3H), 7.10-7.18 (dd, 3H), 7.27-7.34 (m, 6H), 7.77 (s, 2H), 7.82-7.86 (dd, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ ppm: 47.65, 112.91, 115.63, 120.15, 121.05, 123.93, 126.69, 127.36, 1237.86, 129.14, 129.65, 132.17, 135.77, 136.39, 140.10, 143.63, 160.14, 162.56; Mass 408.11 $(M+1)^+$.

1-(2-Methoxynaphthalen-1-yl)methylene)-2-tosylhydrazine (6)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.37 (s, 3H), 3.6 (s, 3H), 7.4-7.46 (m, 3H), 7.66 (s, 1H), 7.7-7.76 (m, 6H), 8.19 (s, 1H), 11.7 (s, 1H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ ppm 56.46, 113.09, 113.54, 123.89, 125.00, 127.76, 128.55, 129.64, 130.33, 132.59, 136.17, 143.51, 144.61, 157.31; Mass 355.11 $(M+1)^+$.

(9H-Fluoren-2-yl)methylene)-2-tosylhydrazine (7)

^{1}H NMR (400 MHz) δ ppm: 2.39 (s, 3H), 3.98 (s, 2H), 7.4 (d, 3H), 7.6 (s, 2H), 7.85 (m, 6H), 11.45 (s, 1H); ^{13}C NMR (100.6 MHz) δ ppm: 20.93, 36.25, 120.15, 123.04, 125.14, 126.13, 126.83, 127.22, 129.64, 132.21, 136.29, 140.31, 142.96, 143.44, 143.59, 147.40; IR (KBr) cm^{-1}: 3194, 2970, 2927, 1595; Mass: 363.2 $(M+1)^+$.

α-Amylase inhibitory activity

Antidiabetic activity of the synthesized compounds was determined in terms of % α-amylase inhibition using the literature method.[28] Starch solution (0.5 % w/v) was prepared using 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.9; 25 mL) in a boiling water

bath for 15 minutes. The α-Amylase solution was prepared by mixing 1U mL^{-1} of α-amylase in the same buffer. The colorimetric reagent was prepared by mixing an equal volume of sodium potassium tartrate tetrahydrate solution and 96 mM 3,5-dinitrosalicylic acid (DNS) solution. Starch solution (1000 µL) was mixed with increasing concentration of an enzyme inhibitor. 1000 µL of the α-amylase solution was added to the synthesized compounds at different concentrations (20, 40, 60, 80, and 100 µg mL^{-1}) or acarbose (20-100 µg mL^{-1}) and incubated at 25 °C for 3 minutes to react with the starch solution. A 1000 µL of 96 mM DNS reagent was added to the above solution and the contents were heated for 15 minutes in the boiling water bath. The final volume was made up with distilled water and absorbance was measured at 540 nm using a spectrophotometer. The percentage inhibition and 50 % inhibitory concentration (IC_{50}) values were then determined (Table 2).

Molecular Docking

Molecular docking study was performed to explore binding affinity, binding mode and possible molecular interactions of the synthesized compounds against the active site of the α-amylase enzyme and the results were expressed in terms of binding energies. The crystal structure of human pancreatic α-amylase complexed with Acarbose ((PDB: 2QV4) downloaded from www.rcsb.org was used for docking studies. Autodock Vina[29] was used to perform docking simulations. Marvin Sketch 5.6.0.0 (2011) was used to draw 2D structures of the compounds which were converted into 3D geometry using the same interface. The geometry of 3D molecules was optimized through energy minimization using UCSF Chimera 1.8[30] during which Gasteiger charges were added and energy minimization was carried out with the combination of steepest descent and conjugate gradient geometry search criteria until gradient converses to 0.05 and 0.01 respectively. The protein was processed by removing water and other nonstandard residues. The resulting clean protein was further optimized by energy minimization in UCSF Chimera with the Amber ff12SB force field and similar geometry search criteria. During the docking simulation, polar hydrogen was added to protein structure with MGLtools1.5.4. All torsion angles for the compounds were set free to perform flexible docking. Grid box of size 18 x 18 x 18 with 1A0 spacing with *x*, *y* and *z* centers 12.94, 47.17 and 26.2 respectively was chosen, which was large enough to cover the active site of the protein. The results of docking simulations were analyzed in terms of estimated binding free energy in kcal mol^{-1} and interactions of ligands with residues at the active site.

CONCLUSION

In summary, we synthesized various Schiff's bases of p-toluenesulphonyl hydrazide using Eaton's reagent (5 mol %) under solvent-free conditions. The synthesis is simple, solvent-free, beneficial and economical from the commercial point of view. The Schiff's bases **1**, **3**, **5** and **6** exhibited good amylase inhibitory activity and high free binding energies; but less than the standard Acarbose at different concentrations 20, 40, 60, 80 and 100 µg mL^{-1} highlighting the future scope in the development of p-toluenesulphonyl hydrazide based compounds as possible diabetic inhibitors as per the pharmaceutical requirement.

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