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Synthesis, *In-vitro* Antioxidant, Anti-diabetic Evaluation and Docking Studies of Newly Synthesized Benzoxazole Derivatives

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Abstract

In the present study, a set of different benzoxazole derivatives has been synthesized from ethyl acetoacetate, ethoxymethylene malononitrile, $NaNO_2$, and organic acids. Analytical instruments like proton NMR (^1H), carbon NMR (^{13}C), infrared spectroscopy (IR), and LC-MS mass spectrometry were used for structural characterization. Synthesized molecules were evaluated for *In-vitro* antioxidant property (DPPH assay, Total antioxidant & reducing power method) and anti-diabetic property (alpha-amylase & alpha-glucosidase assay). *In silico,* studies against Human pancreatic alpha-amylase (PDB ID: 3BAW) have been carried out to get the binding approach of the ligand towards the protein. The results demonstrated that compounds namely **5b**, **6b**, **3b** and **4b** had potent antioxidant and anti-diabetic activity compared with ascorbic acid and acarbose.

Keywords: Benzoxazole, Ethoxymethylene malononitrile, Antioxidant, Anti-diabetic, Molecular docking

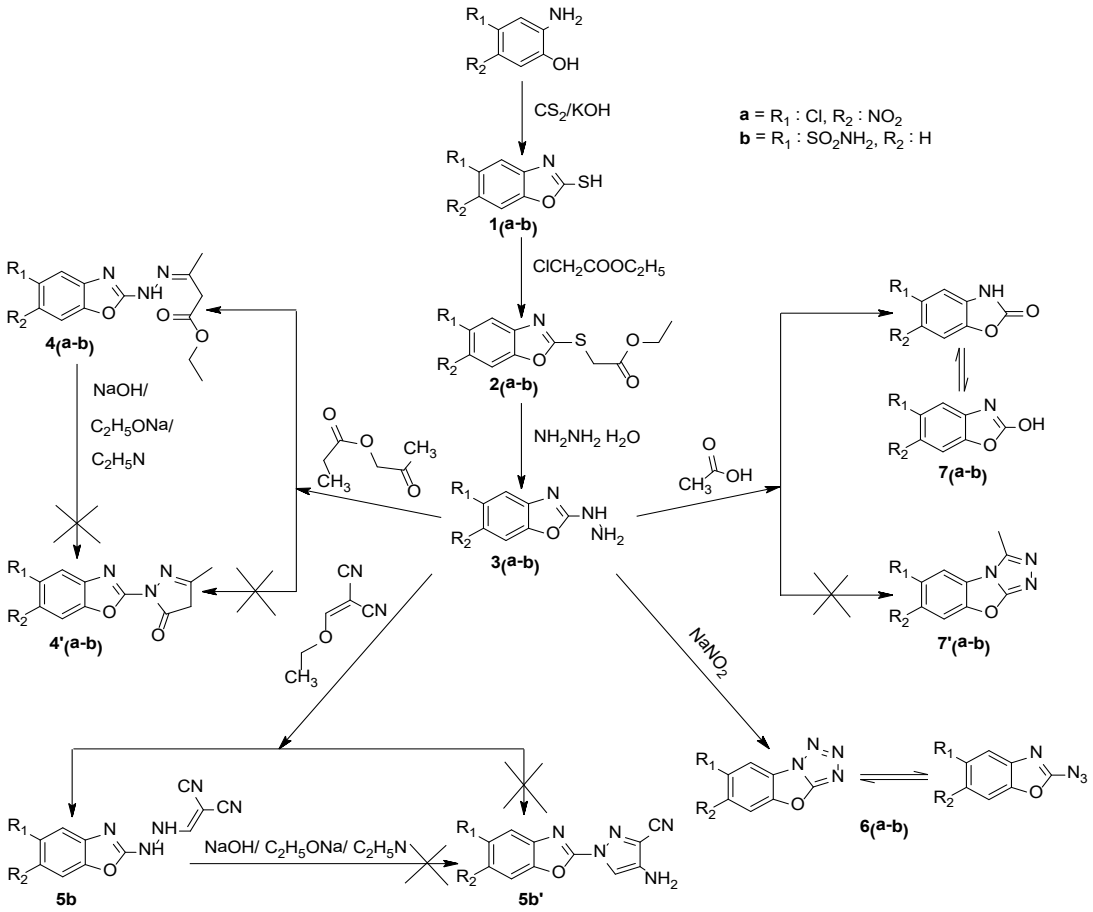
Introduction

Since diabetes has become one of the common problems of human beings, the researchers aimed to find novel methods to prevent and treat it. Although diabetes is caused by a corrupted immune system that destroys the pancreas cells to make proper insulin production, recent research shows that oxidative stress can also be the reason for less secretion of insulin [1]. Hence it is proven that diabetes can be managed with the use of the proper antioxidants.

Even though many molecules in heterocyclic compounds have antioxidant and antidiabetic activity, benzoxazole is one of the most potent moieties for biological evaluation, including both antioxidant [2] and anti-diabetic [3] assay. Literature survey also revealed that the substitution like sulfonamide group shows a variety of biological activities like anti-microbial, anti-inflammatory [4], antioxidant [5], anti-diabetic [6], and anticancer [7] activities. The substitutions like chloro [2], nitro [2] also have good biological activities.

The considerable biological activities of benzoxazole [3,8,9] compounds in drug discovery and their importance in the medicinal field have stimulated the present investigation on the synthesis of derivatives of this ring system along with the substitutions like sulfonamide, nitro and chloro. Hence, it was planned to synthesize fused benzoxazole derivatives. However, the stability of the intermediate formed during the reactions are so high that the attempts to cyclize the molecule by cyclizing agents such as NaOH, pyridine, C_2H_5ONa were not successful as depicted in **Scheme 1**. This is attributed to the fact that the molecules are small ligands and the literature survey has shown remarkable biological activity [10] for such molecules. The functionalities like sulfonamide [7], nitriles [11], tetrazole [12], halogens [13], nitro [14] groups as well as ester [15] derivatives of compounds have shown effective biological activities. Hence, synthesized molecules were exposed for *in-vitro* antioxidant and anti-diabetic investigation.

The synthesized molecules were screened for *in silico* anti-diabetic studies against *Human pancreatic alpha-amylase* (PDB ID: 3BAW). *In silico,* studies of synthesized molecules play a key role in predicting the best conformation considering the lowest binding energy and the number of hydrogen bonds. The results of *In silico* method were compared with the *In-vitro* studies.



Scheme 1 Synthesis of 5-chloro-6-nitro and 5-sulfamoyl substituted benzoxazole derivatives.

Materials and methods

Chemistry

TMS was used as an internal standard for ^{1}H and ^{13}C NMR spectral analysis with δ values as ppm. NMR spectra was recorded on Bruker 400 MHz spectrometer MIT, MAHE, Manipal, Karnataka, India and IISc, Bangalore, Karnataka, India. LC-MS was recorded on LCMS 2010A, SHIMADZU, JAPAN with C18 column and rate of flow 0.2 mL/min using ESI (electron spray ionization) method. Bruker (ALPHA) Platinum ATR Fourier transformed infrared (FT-IR) spectrophotometer was used for recording IR spectra. 230e400 mesh-sized silica gel was used for column chromatography. Electrical melting point apparatus was used for determining melting point. VarioMICRO V1.7.0 (Elemental analysersysteme GmbH) was used for Elemental analysis. For TLC analysis, silica gel 60 GF_{254} (Merck) plates were used, and spots were analyzed by UV light of wavelength 254 nm.

5-chloro-6-nitro and 5-sulfamoyl substituted 1,3-benzoxazole-2-thiol (1a) and (1b)

KOH (2.8 g, 0.05 mol) & 60 mL of methanol was taken in a 100 mL round-bottomed flask. To this, CS_2 (3 mL, 0.05 mol) was added dropwise in ice-cold condition (0 - 5 °C) with constant stirring for few minutes, followed by the addition of 4-chloro-5-nitro-2-aminophenol/opsamide (1.8 g, 0.01 mol). The reaction mixture was refluxed for 3 h 30 min and 5 h 30 min respectively and poured to crushed ice, acidified with dilute acetic acid (pH 6.0) with stirring which gives, **1a** as orange-red solid (1.95 g, 84.56 %), mp 189 - 191 °C and **1b** as brownish solid (1.82 g, 79.06 %), mp 193 - 195 °C, respectively. **5-chloro-6-nitro-1,3-benzoxazole-2-thiol (1a):** IR (ν_{max} cm^{-1}): 3050, 1618, 1511, 661. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 8.14 (1H, s), 7.42 (1H, s). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 115.1, 118.4, 124.9, 140.5, 145.7, 157.5 and 180.2 (C-SH). MS (LCMS): *m/z* 231 (M^{+}), 233 (M^{+2}). Anal. calcd for $C_7H_3ClN_2O_3S$ (230.62): C, 36.45; H, 1.31; N, 12.15. Found: C, 36.34; H, 1.26; N, 12.08. **5-sulfamoyl-1,3-benzoxazole-2-thiol (1b):** IR (ν_{max} cm^{-1}): 3150, 1331. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.454 (2H, s, sulfonamide

NH_2, disappeared on D_2O exchange), 7.611 - 7.616 (1H, dd, *J* 1.7544 Hz, *J* 0.4649 Hz), 7.653 - 7.675 (1H, d, *J* 0.4282), 7.709 - 7.713 (1H, d, *J* 1.7846), 14.159 (1H s, SH, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 108.4, 110.7, 122.2, 132.0, 141.7, 150.3 (C benzoxazole) and 181.5 (C-SH). MS (LCMS): *m/z* 230 (M^+). Anal. calcd for $C_7H_6N_2O_3S_2$ (230.26): C, 36.51; H, 2.63; N, 12.17. Found: C, 36.42; H, 2.55; N, 12.08.

5-chloro-6-nitro and 5-sulfamoyl substituted ethyl [(1,3-benzoxazol-2-yl)sulfanyl]acetate (2a) and (2b)

Marcapto of substituted benzoxazole (2.3 g, 0.01 mol) (1a/1b) was taken in a 100 mL round-bottomed flask containing dry acetone (40 mL) and anhydrous potassium carbonate (3g) followed by the addition of ethyl chloroacetate (1.1 mL 0.01 mol). It was refluxed for 30 min and poured to crushed ice with constant stirring, which gave, **2a** as pale grey color solid (3.0 g, 94.94 %), mp 110 - 112 °C and **2b** as pale pink solid (3.1 g, 98.1 %), mp 140 - 142 °C, respectively. **ethyl [(5-chloro-6-nitro-1,3-benzoxazol-2-yl)sulfanyl]acetate (2a)**: IR (ν_{max} cm^{-1}): 817, 1311, 1484, 1741. ^1H NMR (500 MHz, $CDCl_3$): δ_H 8.02 (1H, s), 7.70 (1H, s), 4.11 (2H, s, S-CH_2), 4.23 - 4.28 (2H, q, *J* 7.1352 Hz, CH_2 ester), 1.28 - 1.31 (3H, t, *J* 7.1269 Hz, -CH_3). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 15.1 (-CH_3), 36.4 (S-CH_2), 60.5 (-OCH_2-), 111.1, 114.7, 126.7, 139.2, 144.2, 156.1, 164.9 and 172.8 (C=O). MS (LCMS): *m/z* 317 (M^+), 319 (M+2). Anal. calcd for $C_{11}H_9ClN_2O_5S$ (316.71): C, 41.71; H, 2.86; N, 8.84. Found: C, 41.62; H, 2.78; N, 8.72. **ethyl [(5-sulfamoyl-1,3-benzoxazol-2-yl)sulfanyl]acetate (2b):** IR (ν_{max} cm^{-1}): 1191, 1327, 1732. ^1H NMR (400 MHz, DMSO-d_6): δ_H 8.01 (1H, d, *J* 1.2059), 7.843 (1H, s), 7.79 (1H, d, *J* 1.7694), 4.325 (2H, s, S-CH_2), 4.148-4.201 (2H, q, *J* 7.0983 Hz, CH_2 ester), 1.185 - 1.221 (3H, t, *J* 7.0979 Hz, CH_3), 7.424 (2H, s, sulfonamide NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 14.2 (CH_3), 35.1 (S-CH_2), 61.5 (OCH_2), 110.1, 115.5, 125.4, 138.6, 145.2, 155.2, 165.7 and 170.7 (C=O). MS (LCMS): *m/z* 316 (M^+). Anal. calcd for $C_{11}H_{12}N_2O_5S_2$ (316.35): C, 41.76; H, 3.82; N, 8.86. Found: C, 41.67; H, 3.76; N, 8.79.

5-chloro-6-nitro and 5-sulfamoyl substituted 2-hydrazinyl-1,3-benzoxazole (3a) and (3b)

Ester derivatives of substituted benzoxazole (3.16g, 0.01 mol) (2a)/(2b) was taken in a 250 mL round-bottomed flask containing methanol (100 mL) followed by the addition of hydrazine hydrate (2.5 mL, 0.05 mol), which were refluxed for 30 min and stirred for 30 min, respectively. Reaction mixture was poured on to crushed ice with constant stirring gives, **3a** as brown solid (1.95 g, 85.3 %), mp 250 - 252 °C and **3b** as purple-violet color solid (1.96 g, 85.74 %), mp 220 - 222 °C, respectively. **2-hydrazinyl-5-chloro-6-nitro-1,3-benzoxazole (3a):** IR (ν_{max} cm^{-1}): 622, 1565, 1641, 3250. ^1H NMR (200 MHz, DMSO-d_6): δ_H 8.23 (1H, s), 7.51 (1H, s), 9.73 (1H, s, NH, disappeared on D_2O exchange), 4.82 (2H, s, NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 110.2, 118.6, 130.8, 142.5, 148.2, 159.6 and 168.9 (C-NH). MS (LCMS): *m/z* 229 (M^+), 231 (M^{+2}). Anal. calcd for $C_7H_5ClN_4O_3$ (228.59): C, 36.78; H, 2.20; N, 24.51. Found: C, 36.68; H, 2.14; N, 24.46. **2-hydrazinyl-5-sulfamoyl-1,3-benzoxazole (3b):** IR (ν_{max} cm^{-1}): 1308, 3000, 3400. ^1H NMR (400 MHz, DMSO-d_6): δ_H 7.646 (1H, d, *J* 1.2935 Hz), 7.503 (1H, s), 7.4903 (1H, d, *J* 1.71162 Hz), 9.102 (1H, s, NH, disappeared on D_2O exchange), 4.615 (2H, s, NH_2, disappeared on D_2O exchange), 7.260 (2H, s, 2H, sulfonamide NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 110.5, 117.5, 120.3, 136.8, 142.1, 152.5 and 167.7 (C-NH). MS (LCMS): *m/z* 228 (M^+). Anal. calcd for $C_7H_7ClN_4O_3S$ (262.67): C, 32.01; H, 2.69; N, 21.33. Found: C, 31.95; H, 2.65; N, 21.23.

5-chloro-6-nitro and 5-sulfamoyl substituted ethyl-3-[2-(1,3-benzoxazol-2-yl) hydrazinylidene] butanoate (4a) and (4b)

Substituted 2-hydrazinyl-1,3-benzoxazole (2.286 g, 0.01 mol) (3a)/(3b) was taken in a 250 mL round-bottomed flask containing ethanol (80 mL) then, ethylacetoacetate (1.4 mL, 0.01 mol) was added and refluxed for 4 and 3 h, respectively. The reaction mixture was poured on to crushed ice with constant stirring, which gave, **4a** as light brown solid (2.5 g, 73.51 %), mp above 285 - 287 °C and **4b** as orange red solid (2.43 g, 71.45 %), mp 292-294 °C respectively. **ethyl-3-[2-5-chloro-6-nitro(1,3-benzoxazol-2-yl)hydrazinylidene] butanoate (4a):** IR (ν_{max} cm^{-1}): 1529, 590, 1658, 1710, 1251. ^1H NMR (400 MHz, DMSO-d_6): δ_H 8.378 (1H, s), 7.677 (1H, s), 3.421 (2H, s, CH_2-C=O), 4.096 - 4.149 (2H, q, *J* 7.131 Hz, CH_2 ester), 1.195 - 1.230 (3H, t, *J* 7.124 Hz, CH_3), 2.034 (3H, s, CH_3), 11.632 (1H, s, NH, disappeared on D_2O exchange) . ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 14.9 (CH_3), 17.4 (CH_3), 43.7, 61.0 (OCH_2), 112.8, 116.6, 124.8, 140.5, 145.7, 152.1, 154.6, 157.5 and 168.1 (C=O). MS (LCMS): *m/z* 341 (M^+), 343 (M^{+2}). Anal. calcd for $C_{13}H_{13}ClN_4O_5$ (340.71): C, 45.83; H, 3.85; N, 16.44. Found: C, 45.78; H, 3.80; N, 16.37. **ethyl-3-[2-5-sulfamoyl(1,3-benzoxazol-2-yl)hydrazinylidene]butanoate (4b):** IR (ν_{max} cm^{-1}): 1312,

1649, 1239. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.335 - 7.838 (3H, m, aromatic), 2.187 (2H, s, CH_2-C=O), 4.012 - 4.135 (2H, q, *J* 7.0983 Hz, CH_2 ester), 2.014 - 2.107 (3H, t, *J* 7.0979 Hz, CH_3), 1.239 (3H, s, CH_3), 11.177 (1H, s, NH, disappeared on D_2O exchange), 7.450 (2H, s, sulfonamide NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 14.5 (CH_3), 17.2 (CH_3), 45.1, 62.0 (OCH_2), 110.8, 120.2, 124.7, 138.5, 145.6, 150.1, 152.4, 154.5 and 172.2 (C=O). MS (LCMS): *m/z* 340 (M^+). Anal. calcd for $C_{13}H_{16}N_4O_5S$ (340.35): C, 45.88; H, 4.74; N, 16.46. Found: C, 45.81; H, 4.69; N, 16.38.

2-[2-(2,2-dicyanoethenyl)hydrazinyl]-1,3-benzoxazole-5-sulfonamide (5b)

5-sulfamoyl substituted 2-hydrazinyl-1,3-benzoxazole (2.286 g, 0.01 mol) (3b) and ethoxymethylene malononitrile (1.22 g, 0.01 mol) in dimethylformamide (65 mL) were refluxed for 2 h. The reaction mixture was allowed to stand for few minutes and then poured onto crushed ice gives, **5b** as pista greenish solid (2.5 g, 82.16 %), mp 330 - 332 °C. IR (ν_{max} cm^{-1}): 2221, 3241, 1324. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.89 (1H, d, *J 8.3648*, CH), 8.01 (1H, d, *J* 8.5249), 8.12-8.22 (4H, m), 7.52 (2H, s, sulfonamide NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 56.5 ($C(CN)_2$), 111.2, 115.3 (CN), 119.7, 123.5, 135.4, 141.2, 151.6, 165.4 and 172.5. MS (LCMS): *m/z* 305 (M^+). Anal. calcd for $C_{11}H_8N_6O_3S$ (304.28): C, 43.42; H, 2.65; N, 27.62. Found: C, 43.35; H, 2.58; N, 27.55.

7-chloro-6-nitro and 7-sulfamoyl tetrazolo[5,1-*b*][1,3]benzoxazole (6a) and (6b)

Substituted 2-hydrazinyl-1,3-benzoxazole (2.286 g, 0.01 mol) (3a)/(3b) **was** taken in a 250 mL round-bottomed flask containing glacial acetic acid (65 mL) maintaining the temperature at −5 °C by keeping it in ice bath. Then, sodium nitrite (1.035 g, 0.015 mol) was added to the solution dropwise. The reaction mixture was stirred for 30 min. The reaction mixture was poured on to crushed ice with constant stirring, which gave, **6a** as yellowish brown solid (1.92 g, 80.33 %), mp 182 - 184 °C and **6b** as brown solid (1.90 g, 79.5 %), mp 180 - 182 °C**,** respectively. **7-chloro-6-nitro-tetrazolo[5,1-b][1,3]benzoxazole (6a):** IR (ν_{max} cm^{-1}): 2185, 1524. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 8.622 (1H, s), 8.108 (1H, s). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 110.2, 116.6, 124.9, 139.4, 145.2, 152.6 and 159.3. MS (LCMS): *m/z* 339 (M^+), 341 (M^{+2}). Anal. calcd for $C_7H_2ClN_5O_3$ (239.57): C, 35.09; H, 0.84; N, 29.23. Found: C, 35.05; H, 0.80; N, 29.18. **7-sulfamoyl-tetrazolo[5,1-b][1,3]benzoxazole (6b):** IR (ν_{max} cm^{-1}): 2140, 1608, 1356. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.82-8.02 (3H, m), 7.47 (2H, s, sulfonamide NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 111.8, 118.7, 123.7, 138.3, 142.4, 151.6 and 154.2. MS (LCMS): *m/z* 339 (M^+). Anal. calcd for $C_7H_5N_5O_3S$ (239.21): C, 35.15; H, 2.11; N, 29.28. Found: C, 35.08; H, 2.05; N, 29.22.

5-chloro-6-nitro and 5-sulfamoyl substituted 1,3-benzoxazol-2(3H)-one (7a) and (7b)

Substituted 2-hydrazinyl-1,3-benzoxazole (2.286 g, 0.01 mol) (3A)/(3B) was taken in a 100 mL round-bottomed flask. A 40 mL of formic acid/acetic acid was added along with a few drops of concentrated hydrochloric acid. It was refluxed for 3 h. The reaction mixture was allowed to stand for few minutes and then it was poured onto crushed ice giving, **7a** as yellowish green solid (1.93 g, 89.95 %), mp 235 - 237 °C and **7b** as brown solid (1.95 g, 90.88 %), mp 242 - 245 °C, respectively. **5-chloro-6-nitro-1,3-benzoxazol-2(3H)-one (7a):** IR (ν_{max} cm^{-1}): 3260, 2918, 1767, 1564, 813. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.422 to 8.288 (2H, m, aromatic), 11.40 (1H, s, NH, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 120.31, 124.32, 126.48, 135.27, 144.11, 148.27 (benzoxazole) and 155.54 (C=O). MS (LCMS): *m/z* 215 (M^+), 217 (M^{+2}). Anal. calcd for $C_7H_3ClN_2O_4$ (214.56): C, 39.18; H, 1.41; N, 13.06. Found: C, 39.14; H, 1.38; N, 13.02. **5-sulfamoyl-1,3-benzoxazol-2(3H)-one (7b)**: IR (ν_{max} cm^{-1}): 3180, 1750. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.441-7.578 (3H, m, aromatic), 12.01 (1H, s, NH, disappeared on D_2O exchange), 7.38 (2H, s, sulfonamide NH_2, disappeared on D_2O exchange). ^{13}C NMR (400 MHz, DMSO-d_6): δ_c 121.35, 123.63, 124.69, 131.54, 133.11, 138.72 (C benzoxazole) and 141.45 (C=O). MS (LCMS): *m/z* 215 (M^+). Anal. calcd for $C_7H_6N_2O_4S$ (214.19): C, 39.25; H, 2.82; N, 13.08. Found: C, 39.21; H, 2.79; N, 13.03.

Results and discussion

In silico studies

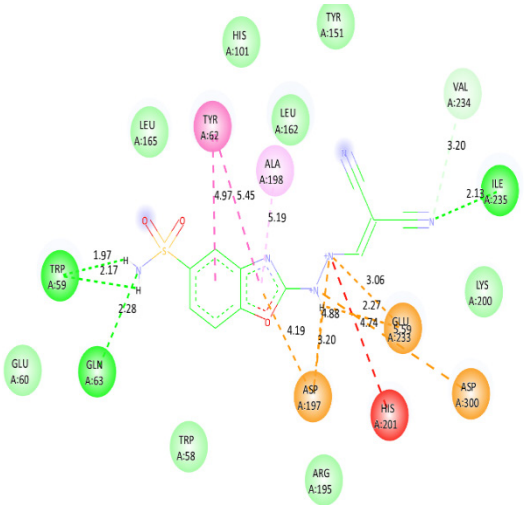
The newly synthesized benzoxazole nucleus derivatives (1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5b, 6a, 6b, 7a and 7b) were docked against target *Human pancreatic alpha-amylase* (PDB ID: 3BAW) [16] using AutoDoc 4.2 platform. The binding energies of the same is tabulated in **Table 1**. The study was made to find the possible binding mode and interaction of molecules with amino acid residues. Initial structure optimization and energy minimization of all ligands were carried out using OpenBabel 2.3.2 [17],

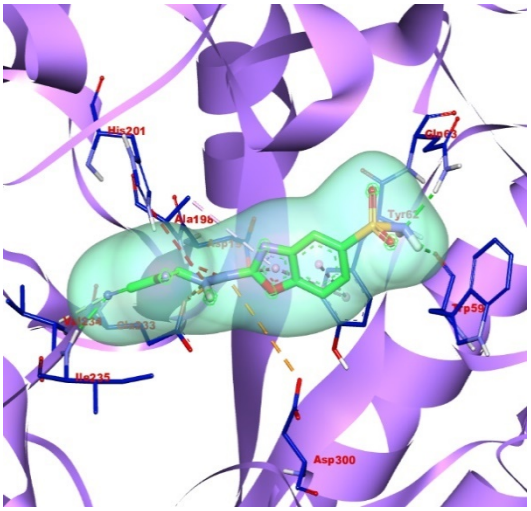
followed by the addition of Gasteiger charges along with polar hydrogen atoms. An objective-based docking technique was utilized in this investigation to set up the grid maps framework. The lattice points of the grid box (spacing 0.375) and grid centre of maps was set at X = 58, Y = 48, Z = 48 and x = 14.919, y = −17.939, z = −22.527, respectively. ADT scoring function was used for the determination of Ligand binding affinity, and it was anticipated as negative Gibbs free energy (ΔG) (Kcal/mol). On the basis of the binding energy and the formation of hydrogen bonds, the nature of the interaction, whether strong, medium or low, was determined. The posture with the highest negative value was regarded as the best conformation. DS Visualizer (v21.1.0.20298) has been used to perform post-docking analysis.

Table 1 Binding energies by Molecular docking studies to the compounds against Human pancreatic alpha-amylase (PDB ID: 3BAW).

Compound	1a	1b	2a	2b	3a	3b	4a	4b	**5b**	6a	**6b**	7a	7b	**Acarbose**
Binding energy (kcal/mol)	−6.32	−6.04	−7.04	−6.73	−6.12	−6.77	−6.54	−7.08	**−8.12**	−5.98	**−8.00**	−5.15	−6.78	**−11.27**

(a)





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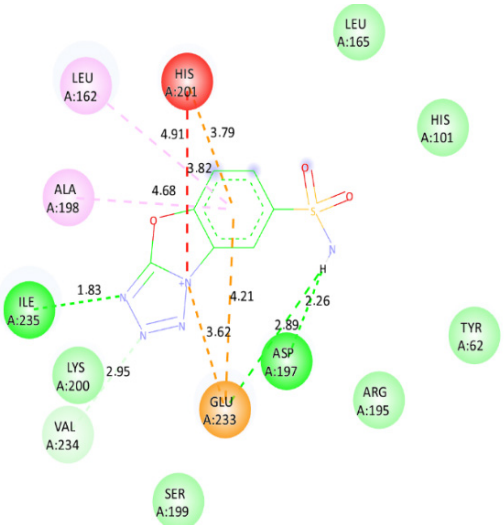

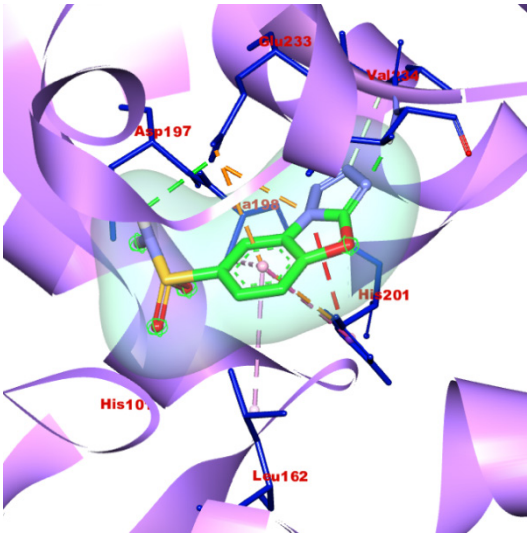

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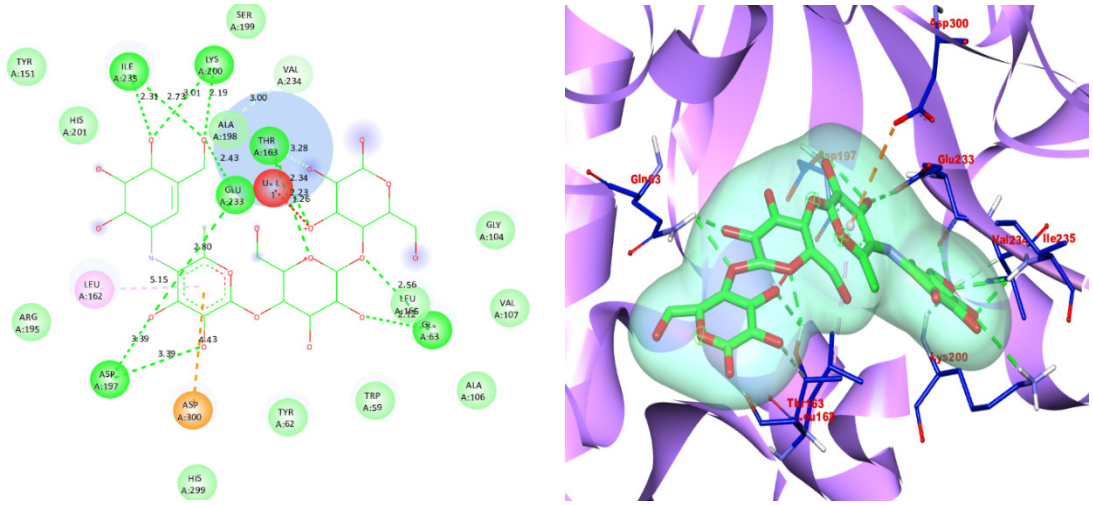

Figure 1 Molecular docking analysis of lead compounds 5b (a) and 6b (b) compared with acarbose (c) against Human pancreatic alpha-amylase.

Among the 13 molecules, it was found that the binding energy of molecules **5b**, **6b**, **3b** and **4b** was the lowest viz, **−8.12**, **−8.00**, **−6.77** and **−7.08** by exhibiting 2, 4, 1 and 3 hydrogen bonds, respectively (**Table 2**). In the active pocket, amino acid residues ASP300, GLN63, THR163, ARG195, ASP197, ILE235, TRP59, GLN63, GLN63, LYS200, GLU233 and THR163 formed the Hydrogen bonds with Human pancreatic alpha-amylase. The hydrogen and hydrophobic interaction profile of molecules **5b** and **6b** with the receptor, and their distances are shown in **Figure 1**. From the present study, it can be suggested that **5b** and **6b** ligand molecules can show better inhibition. In this context, all synthesized molecules were further subjected to anti-diabetic experimental validation to confirm these findings.

Table 2 Molecular docking results of lead compounds compared with acarbose against Human pancreatic alpha-amylase.

Lead compounds	Binding energy (kcal/mol)	Protein-ligand interaction		
		No. of H-bonds	Amino acid residues	Distance (Å)
3b	−6.77	1	ASP300	2.14
4b	−7.08	3	GLN63, THR163, ARG195	2.17, 1.97, 1.66
5b	−8.12	2	ASP197; ILE235	2.26; 1.83
6b	−8.00	4	ILE235; TRP59; GLN63	2.13; 2.17; 1.97; 2.28
Acarbose	−11.27	10	ASP197; GLN63; LYS200; ILE235; GLU233; THR163	3.39; 2.56; 2.12; 2.19; 3.01; 2.73; 2.31; 2.80; 2.34

Biological studies

The synthesized 13 compounds were screened for 3 different varieties of antioxidant activity, and 2 different methodologies were employed for antidiabetic activity. Antioxidant activity was performed by DPPH assay, Total antioxidant & Reducing power method whereas antidiabetic activity was carried out by alpha-amylase & alpha-glucosidase assay.

***In-vitro* antioxidant studies**

DPPH assay: The method by Brand-William *et al*. [18] was used to calculate the compound's radical scavenging behavior against stable 2,2-diphenyl 2-picrylhydrazyl hydrate (DPPH). DPPH was reduced when it came into contact with an antioxidant compound that could donate hydrogen. On a UV visible spectrophotometer, the color transition (from dark violet to pale yellow) was measured at an optical density of 515nm.

The standard reference for the DPPH test was ascorbic acid. Distilled water was used for the preparation of stock solution by ascorbic acid (1 mg/mL; w/v). A 60 µM solution of DPPH in methyl alcohol was freshly prepared and 3.9 mL of this solution was mixed with 100 µL of the test sample at several concentrations (6.25, 12.5, 25, 50 and 100 µg/mL). The test tubes were kept in the dark for 15 minutes at room temperature and then the change in absorbance was measured at 515 nm. The control was made using only DPPH solution, no extract, and no ascorbic acid. 95 % methyl alcohol was used as a blank solution. The radical scavenging behavior was calculated using the formula below.

$$Percentage\ inhibition = \frac{(\text{Absorbance of Control at 0 min} - \text{Absorbance of test}) \times 100}{Absorbance\ of\ control\ at\ 15\ min}$$

Table 3 Absorbance of the synthesized compound at 515 nm for DPPH assay.

Compound / Concentrations	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
	OD at 515 nm				
1a	0.684	0.607	0.489	0.335	0.094
1b	0.700	0.472	0.291	0.092	0.058
2a	0.589	0.520	0.290	0.150	0.093
2b	0.599	0.463	0.288	0.156	0.092
3a	0.687	0.619	0.499	0.290	0.099
3b	0.596	0.447	0.268	0.101	0.085
4a	0.606	0.466	0.358	0.292	0.147
4b	0.598	0.526	0.283	0.154	0.084
5b	0.540	0.407	0.142	0.085	0.055
6a	0.698	0.634	0.517	0.325	0.128
6b	0.553	0.430	0.184	0.120	0.072
7a	0.697	0.640	0.533	0.358	0.119
7b	0.604	0.459	0.294	0.140	0.076
Ascorbic acid	0.629	0.494	0.334	0.066	-
Control at 0 min			0.747		
Control at 15 min			0.701		

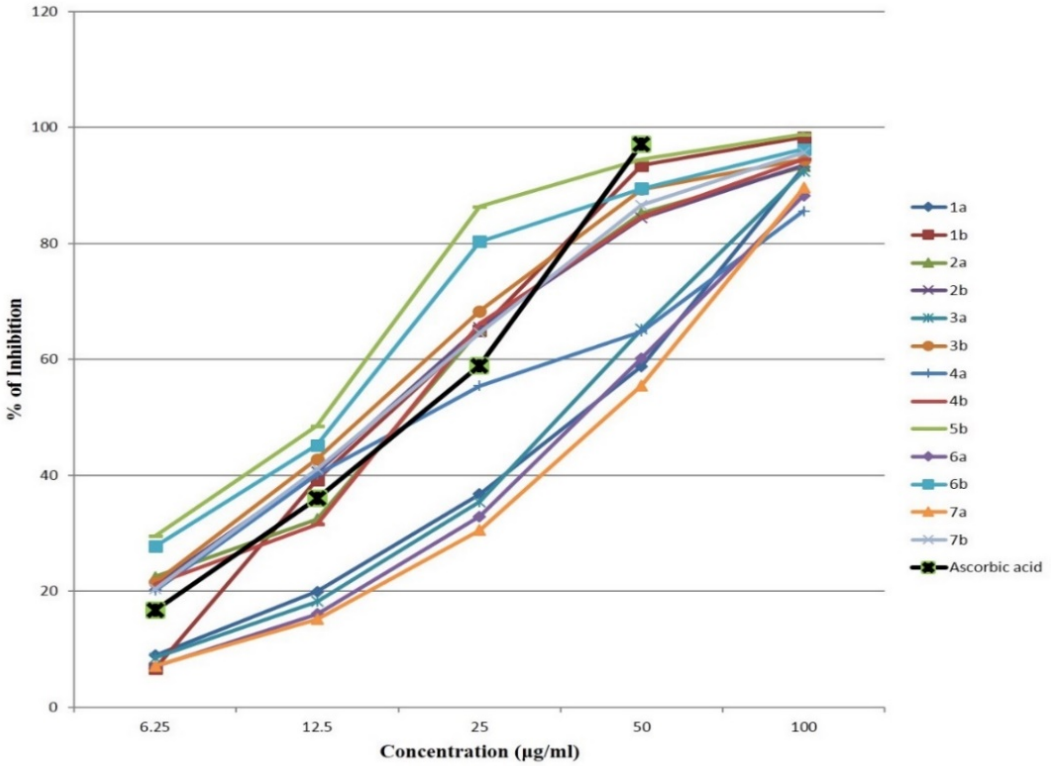

Figure 2 Inhibition of compounds in DPPH assay.

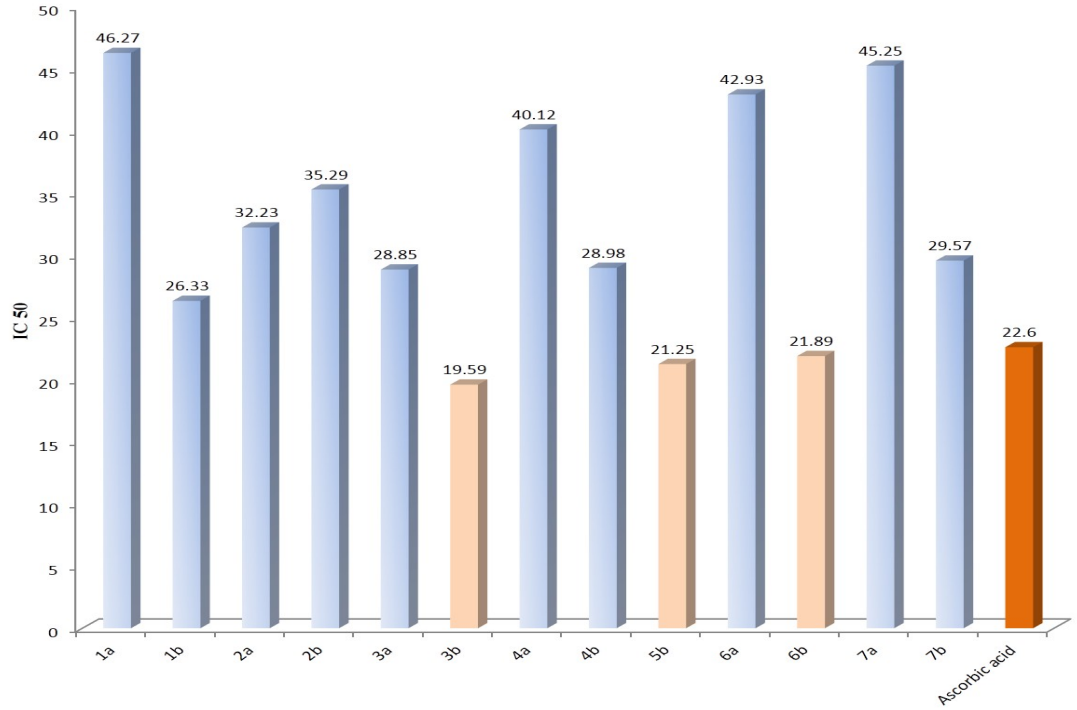

Figure 3 Comparison of IC 50 values of synthesized compounds.

All synthesized compounds have shown the capacity for scavenging the free radical when compared with the standard Ascorbic acid. % of inhibition was plotted against different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) in **Figure 2**. IC 50 values of all the compounds are shown in **Figure 3**. The absorbance (Optical density) for all compounds at 515 nm was tabulated in **Table 3**. With all the above data out of 13 compounds, **5b** and **6b** showed potent activity followed by **4b** and **3b** when compared with standard ascorbic acid.

Total antioxidant activity (phospho-molybdenum test)

The phospho-molybdenum method was used to determine the compound's total antioxidant ability. It is on the basis of compounds reducing Mo (VI) to Mo (V) and then forming a Mo(V) complex of green phosphate at a pH of acidic. It quantifies antioxidants that are both water-soluble and fat-soluble (total antioxidant capacity).

1 mg/mL of sample was mixed with 3 mL of reagent solution (28 mM sodium phosphate, 0.6 M sulfuric acid and 4 mM ammonium molybdate) as per the protocol designated by Prieto *et al*. [19]. The standard was ascorbic acid and 1 mL of different concentrations of the standard were mixed with 3 mL of reagent solution. The reaction solution was incubated for 90 min at 95 °C in test tubes. After cooling at room temperature, the optical density of the solution was measured against a blank with a UV-VIS spectrophotometer at 695 nm. The number of gram equivalents of ascorbic acid is used to measure overall antioxidant activity. Ascorbic acid and methyl alcohol were mixed together to create the calibration curve.

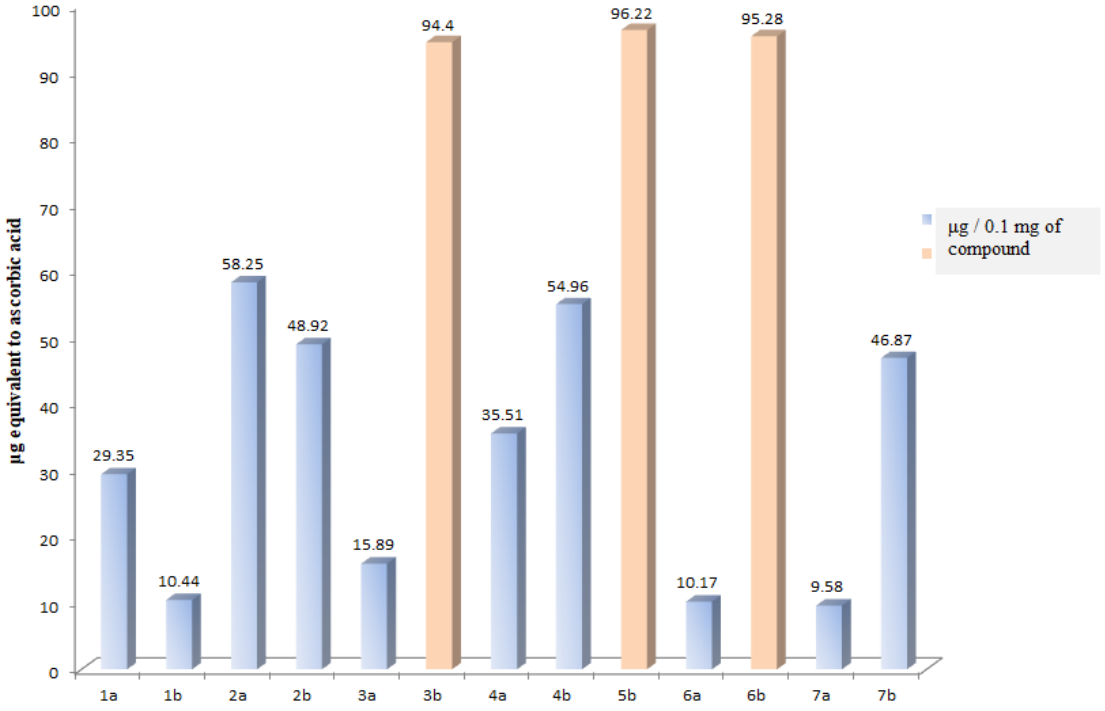

Figure 4 Total antioxidant capacity of compounds.

The total antioxidant capacities are well expressed as gram equivalents of ascorbic acid. Amongst the 13 compounds, **5b**, **6b** and **3b** showed good antioxidant action as equivalents to ascorbic acid. **Figure 4** gives the outcomes of µg equivalent to ascorbic acid to each of the tested compounds.

Reducing power method

The Oyaizu [20] method was used to evaluate the compounds reducing strength. The compounds were combined with 1.25 mL of 1 % potassium ferricyanide and 1.2 mL of sodium phosphate buffer (pH 6.6) at different concentrations (6.25, 12.5, 25, 50 and 100 µg). For 20 min, the mixture was incubated at 50 °C. Upon incubation, 1.25 mL of 10 % trichloroacetic acid (w/v) was added, followed by a 10-min

centrifugation. The absorbance of the top layer (1 mL) was estimated at 700 nm after it was combined with 1 mL of distilled water and 125 µL of 0.1 % ferric chloride (w/v). For set 0, a negative control (Reagent Blank) was used.

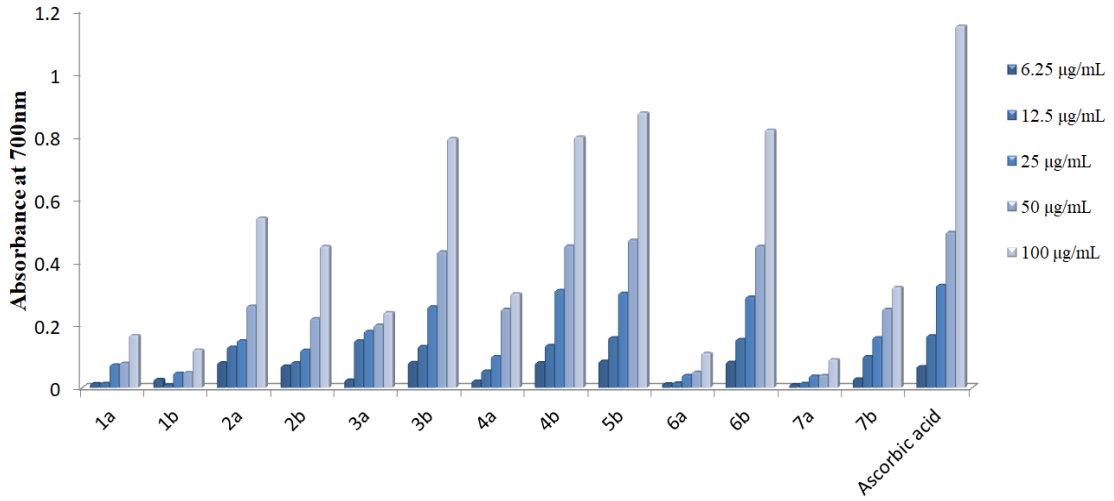

Figure 5 Total reductive capability of compounds.

The reducing powers of the compounds were compared with standard ascorbic acid. Among the 13 tested compounds, compound **5b** showed higher absorbance indicating higher reducing power, followed by **6b**, **4b** and **3b**, whereas **7a** and **6a** showed very little absorbance. The results are given in **Figure 5**.

***In-vitro* Anti-diabetic studies**

α-glucosidase inhibition assay

According to Kim *et al.* [21] procedure, the inhibitory activity of the sample on the α-glucosidase enzyme was determined. For 10 min, 200 µL of diluted α-glucosidase (0.067 U/mL) were pre-incubated with varying concentrations of the sample. In 0.1 M sodium phosphate buffer of pH 6.9, the substrate solution pNPG (p-nitrophenyl α-D-glucopyranoside) was developed. To begin the reaction, 200 µL of 3.0 mM pNPG was prepared as the substrate in 0.1 M sodium phosphate buffer. At 37 °C the reaction solution was incubated for 20 min before being stopped with 2 mL of 0.1 M Na_2CO_3. The yellow-colored para-nitro phenol released from pNPG was measured at 400 nm to determine the α-glucosidase activity.

The percentage of inhibition was used to express the effect on the molecules in the results. The same procedure was done with Acarbose (1 mg/mL) which was used as standard. The % of Inhibitory activity was calculated by the below formula;

Inhibitory activity (%) = (B-T/B-C) ×100

where, B is the absorbance of blank,
T is the absorbance in the presence of test substance,
C is the absorbance of control.

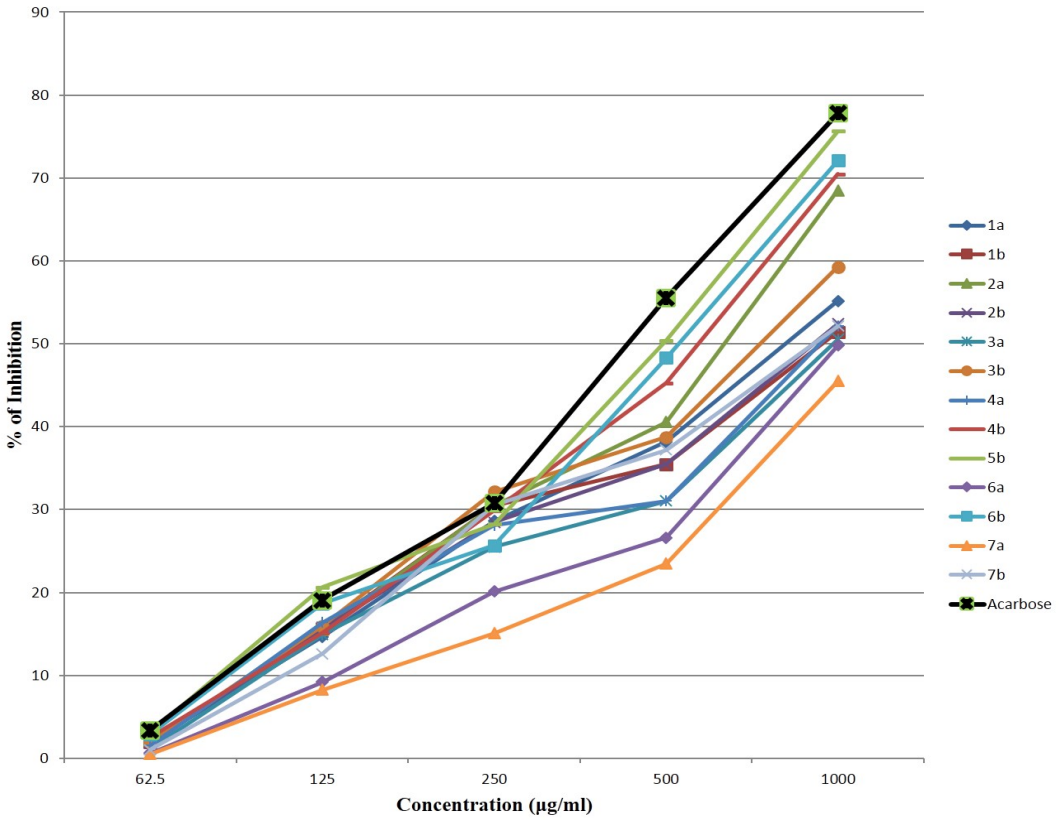

Figure 6 α-glucosidase inhibition of compounds.

In **Figure 6**, % of Inhibition of all synthesized compounds were plotted against different concentrations (62.5, 125, 250, 500 and 1000 µg/mL) and compared with standard drug Acarbose. Compounds absorbance at 400 nm were tabulated in **Table 4**.

Table 4 Absorbance of the synthesized compound at 400 nm for α -Glucosidase inhibition assay.

Compound / Concentrations	**62.5 µg/mL**	**125 µg/mL**	**250 µg/mL**	**500 µg/mL**	**1000 µg/mL**
	OD at 400 nm				
1a	0.855	0.745	0.625	0.542	0.395
1b	0.855	0.737	0.608	0.565	0.427
2a	0.854	0.738	0.608	0.521	0.279
2b	0.856	0.737	0.626	0.565	0.419
3a	0.862	0.743	0.652	0.603	0.435
3b	0.855	0.734	0.594	0.537	0.359
4a	0.859	0.730	0.629	0.603	0.421
4b	0.850	0.741	0.614	0.480	0.263
5b	0.845	0.694	0.628	0.436	0.218
6a	0.867	0.792	0.698	0.642	0.441
6b	0.847	0.711	0.650	0.454	0.248
7a	0.867	0.801	0.741	0.669	0.479
7b	0.863	0.763	0.608	0.550	0.421
Acarbose	0.843	0.707	0.606	0.392	0.199
Blank			0.872		
Control			0.007		

Out of all the compounds tested, **5b** and **6b** showed good inhibition with reference to standard acarbose. However, **7a** and **6a** were less active towards the α-glucosidase inhibition assay when compared with all the tested compounds.

α-amylase inhibitory assay by DNSA method

The anti-diabetic action of the test samples was determined according to the method described in the Worthington Enzyme Manual with slight modifications [22,23]. In brief, 500 µL of 0.02 M Na_3PO_4 buffer of pH 6.9 with 0.006 M NaCl containing 0.5 mg/mL of α-amylase enzyme and different concentrations (in µg) of the test sample as enzyme inhibitor were pre-incubated at 37 °C for 10 min. After the pre-incubation, each test tube was filled with 500 µL of a 1 % starch solution in 0.02 M Na_3PO_4 buffer of pH 6.9 and incubated at room temperature for 5 min. 1.0 mL of dinitrosalicylic acid (DNSA) reagent was used to avoid the reaction.

After the pre-incubation, 500 µL of a 1 % starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube and incubated at room temperature for 5 min. The reaction was stopped using 1.0 mL of dinitrosalicylic acid (DNSA) reagent. The test tubes were heated for 5 min in boiling water and then cooled at ambient temperature. By adding distilled water to the reaction mixture, the volume was increased to 10 mL. The UV-Visible spectrophotometer was used at 540 nm, and the optical density of the compound was measured. The control sample and blank was taken for comparison for the absorbance of test compounds. The percentage of inhibition for the test sample is expressed below;

$$\text{Percentage inhibition} = \frac{(B - A) \times 100}{(B - C)}$$

where, C - Absorbance of the control with starch and without alpha-amylase,
B - Absorbance of the control with starch and alpha-amylase,
A - Absorbance of the test.

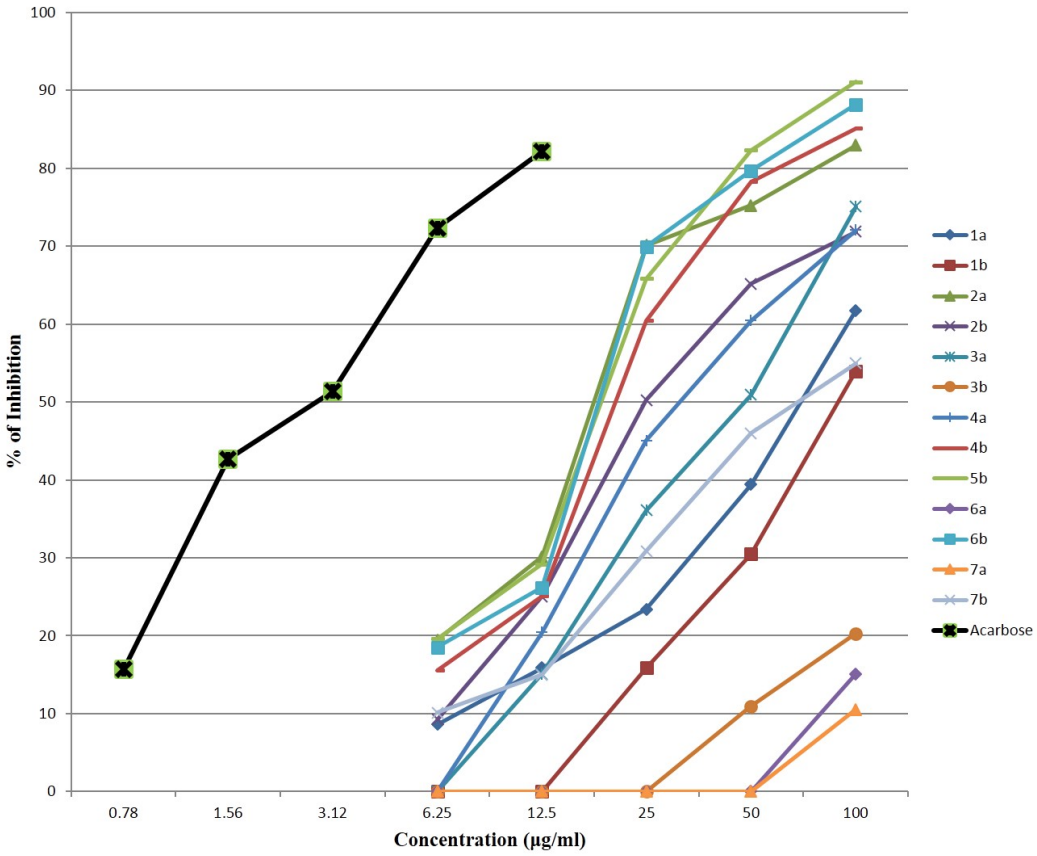

Figure 7 α-amylase inhibitory capacity of compounds.

In the **Figure 7**, % of Inhibition of all synthesized compounds were plotted against different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µg/mL) and compared with standard drug Acarbose.

Table 5 Absorbance of the synthesized compound at 540 nm for α-amylase inhibition assay.

Compound / Concentrations	**6.25 µg/mL**	**12.5 µg/mL**	**25 µg/mL**	**50 µg/mL**	**100 µg/mL**
			OD at 540 nm		
1a	0.994	0.918	0.840	0.673	0.441
1b	-	-	0.918	0.766	0.522
2a	0.881	0.770	0.355	0.301	0.221
2b	0.987	0.823	0.561	0.406	0.336
3a	-	0.926	0.708	0.554	0.303
3b	-	-	-	0.969	0.872
4a	-	0.871	0.615	0.455	0.334
4b	0.921	0.822	0.455	0.270	0.198
5b	0.879	0.780	0.399	0.227	0.137
6a	-	-	-	-	0.926
6b	0.891	0.811	0.356	0.255	0.167
7a	-	-	-	-	0.973
7b	0.978	0.927	0.763	0.605	0.512
Blank			1.083		
Control			0.044		
			OD at 540 nm		
	0.78 µg/mL	**1.56 µg/mL**	**3.12 µg/mL**	**6.25 µg/mL**	**12.5 µg/mL**
Acarbose	0.601	0.439	0.387	0.261	0.202
Blank			0.695		
Control			0.095		

The absorbance of the tested compound at 540 nm has been tabulated in **Table 5**. Out of all the compounds tested, **5b** and **6b** showed a near percentage of inhibition with reference to standard acarbose.

Conclusions

In the present work, biologically important sulfonamide functionality was considered while synthesizing benzoxazole derivatives to evaluate the probability of an increase in potency of the benzoxazole nucleus. As per the expectation, the benzoxazole molecules having sulfonamide functionality stood out in the activities performed.

Among the 13 molecules, **5b**, **6b** and **3b** showed significant results for subjected antioxidant and anti-diabetic activity. In the DPPH assay, below 50 µg/ml, compounds **5b**, **6b** and **3b** outperformed in comparison with the standard ascorbic acid and IC50 values were on par with the standard. The total antioxidant capacity study also showed as the molecules **3b**, **5b** and **6b** were equivalent to ascorbic acid. In anti-diabetic activity i.e., α-glucosidase and α-amylase inhibitory assay, molecule, **5b, 6b** and **4b** emerged as potent anti-diabetic compounds in comparison with the standard acarbose.

The results of *in silico* studies carried out for anti-diabetic assay also compliments the in-vitro studies. The presence of the sulfonamide group is a common factor in **3b**, **4b**, **5b** and **6b** molecules. In comparison with standard drug ascorbic acid and acarbose, these molecules also contain oxygen and nitrogen atoms that are responsible for hydrogen bonding. The small 3-dimensional stable structure increases the permeability and its ability to form hydrogen bonding. The molecule shows good activity towards antioxidant and anti-diabetic. In progress to the current work, the potent molecules are subjected to cytotoxicity and specific organ toxicity.

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