A Facile Single Pot Synthesis of Highly Functionalized Tricyclic Heterocycle Compounds *via* Sequential Knoevenagel-Michael Addition and their α-Glucosidase Inhibition, Antioxidants and Antibacterial Studies

[1-3]Faiz Ahmed, [1]Abdul Rauf*, [2]Ahsan Sharif, [2]Ejaz Ahmad, [2]Muhammad Arshad, [3]Breno Pannia Esposito, [4]Telma Mary Kaneko and [5]Ashfaq Mahmood Qureshi*.

[1]*Department of Chemistry, The Islamia University of Bahawalpur, Bahawalpur, Pakistan, 63100.*

[2]*Institute of Chemistry, University of Punjab, Lahore Pakistan,.*

[3]*Institute of Chemistry, University of São Paulo, 05508-000 São Paulo, Brazil.*

[4]*Faculty of Pharmaceutical Sciences, University of São Paulo, 05508-000 São Paulo, Brazil.*

[5]*Department of Chemistry, The Government Sadiq College Women University, Bahawalpur, Pakistan, 63100.*

lecorganic@yahoo.com, shamashfaq@yahoo.com

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Summary: A facile methodology was developed which involved multicomponent single pot reaction which yielded the synthesis of highly functionalized tricyclic-heterocycle compounds (**1c-6c**) and (**1d-7d**) in good yields (60-80%). The discovered novel methodology involved sequential multicomponent reactions; consisting of Knoevenagel reaction followed by Michael addition; moreover, the proposed mechanism is consistent with the stepwise methodology which also availed the same tricyclic heterocycle compounds (**1c-6c**) and (**1d-7d**). *In-silico* (α-glucosidase inhibitory studies only) and *in-vitro* biological evaluation was extensively performed for all the synthesized compounds. Tricyclic-heterocycle compounds (**1c-6c**) and (**1d-7d**) showed excellent interacting affinity with receptor protein (PDB ID: 3A47) which were further complemented and confirmed through *in-vitro* α-glucosidase inhibitory studies which were found to be comparable with standard acarbose. Whereas, **3c, 3d** and **5d** exhibited IC_{50} value (111.8, 99.4, 108.7 µmol/L) as compared to standard acarbose (135.6 µmol/L) making them excellent α-glucosidase inhibition candidates among the lot. Furthermore, all the novel synthesized compounds were screened for *in-vitro* antibacterial, and antioxidant studies, which revealed that all these compounds show mild bacteriostatic properties at 200 µM concentration against 10^5 CFU/200 µL of three bacterial strains; *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*, while compounds (**2d, 3d, 4d, 6c, 6d**) are found more potent antioxidants than the standard ascorbic acid.

Keywords: Tricyclic Heterocycles, Sequential Knoevenagel-Michael addition, α-glucosidase inhibition, Antioxidants, Antibacterial studies.

Introduction

In academic and industrial research related to organic synthesis there is always quest for greener and efficient reactions or methodologies [1], which can lead to the synthesis of complex target molecules with ease, less complications and side products. In this regard Knoevenagel reaction is one of the unique one due to its simplicity and extensive applications [2], these Knoevenagel adducts are employed as precursor for synthesis of many significant molecules, heterocyclic compounds and important drugs [3]. This reaction is successfully used in various type of domino or cascade reactions, in which two or more subsequent transformations take place on the basis of functionality generated in the preceding step [4]. Moreover, recently this strategy has shown great promise to target heterocyclic compounds using suitable reagents [5]. Heterocyclic compounds always remain in limelight due to their abundance and significance in nature, along with pharmaceutical and medicinal properties [6]. Number of heterocyclic analogues has been reported as therapeutic scaffold with significant activity against cancer [7], microbial pathogens[8], fungal infections, and more challenging diseases such as Alzheimer [9]. Furthermore numerous heterocyclic compounds are known for their antioxidants activity [10] i.e., having radical scavenging activity, they also have been reported as anti-tumour [11-12], antimicrobial [8] and anti-inflammatory agents [13]. It has been speculated that the presence of antioxidant agents along α-glucosidase inhibitory properties in diet and drugs promote healthy life [14]. One of the mammoth hurdle in healthy life style is higher trends of diabetes mellitus across the globe. Although the use of insulin is the predominant line of therapy to control this menace, however, the intake of carbohydrate digesting enzyme inhibitors along with food play a significant role in controlling blood sugar level by plummeting of polysaccharides breakdown into glucose or reducing absorption of glucose in intestine [15-16]. So α-glucosidase inhibition is considered as one of the effective approach to regulating type II

***To whom all correspondence should be addressed.**

diabetes by controlling glucose uptake [14]. Therefore, nowadays in medicinal chemistry *in-silico* and *in-vitro* screening of synthesized molecules against multiple targets is considered as the new approach toward drug design and discovery. Following this approach, herein the synthesis of highly functionalized tricyclic heterocycle compounds is reported along with *in-silico* and *in-vitro* α-glucosidase inhibitory studies. Furthermore, all the synthesized compounds were also screened for *in-vitro* antibacterial and antioxidant screening which will not only interest medicinal chemist but also will attract pure synthetic chemist to employ this novel methodology to target various challenging tricyclic heterocycle targets.

Experimental

Materials and methods

All the chemicals were purchased from Alfa Aesar and Merck and were used without further purification unless stated. TLC Silica gel 60 F_{254} plates were obtained from Merck. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 300 MHz spectrometer using DMSO-d_6 as solvent. Chemical shifts are expressed in parts per million (ppm) and coupling constants in Hertz (Hz). Mass spectrum were analysed on Amazon Speed ETD-Bruker Daltonics, while HRMS analyses were performed on MicroToF Bruker Daltonics. Elemental analysis was recorded on Perkin Elmer 2400 series II. Melting point (m.p.) was recorded on Gallen-Kamp apparatus and was uncorrected.

Synthesis

General Procedure for single pot synthesis of Tricyclic heterocycles (**1c-6c**) *and* (**1d-7d**)**.**

In 100 mL round bottom flask barbituric acid/thiobarbituric acid **8a/8b** (0.005 mol, 2 equivalent) and aromatic aldehydes **1-7**, (0.0025 mol, 1 equivalent) was stirred in 25 mL of absolute ethanol, and catalytic amount of sodium ethoxide was added to the reaction mixture. Reaction mixture was refluxed 4-6h and course of reaction was monitored through TLC. Upon completion of reaction the reaction mixture was cooled down and solvent was evaporated and, dissolution of the crude product was done in distilled water 20 mL. Which upon on acidifying with few drops of diluted HCl yielded the desired product, which was collected through *vacuum* filtration, further washed with hot ethanol to remove impurities and finally afforded the products (**1c-6c**) and (**1d-7d**) in good yield (60-70%) (**Scheme 1**).

General Procedure for synthesis of Knoevenagel adducts **(1a-7a)** *and* **(1b-7b).**

Equimolar amounts of barbituric acid/thiobarbituric acid, (0.0025 mole, 1equivalent) and subsequent aryl aldehydes **1-7**, (0.0025 mol, 1 equivalent) was charged in 100 mL round bottom flask and refluxed in 20 mL dry ethanol till the completion of the reaction and the precipitate obtained were collected through vacuum filtration, solid product obtained was washed with hot ethanol to remove impurities which finally afforded the desired products (**1a-7a**) and (**1b-7b**) in solid state with excellent yield (80-95%).

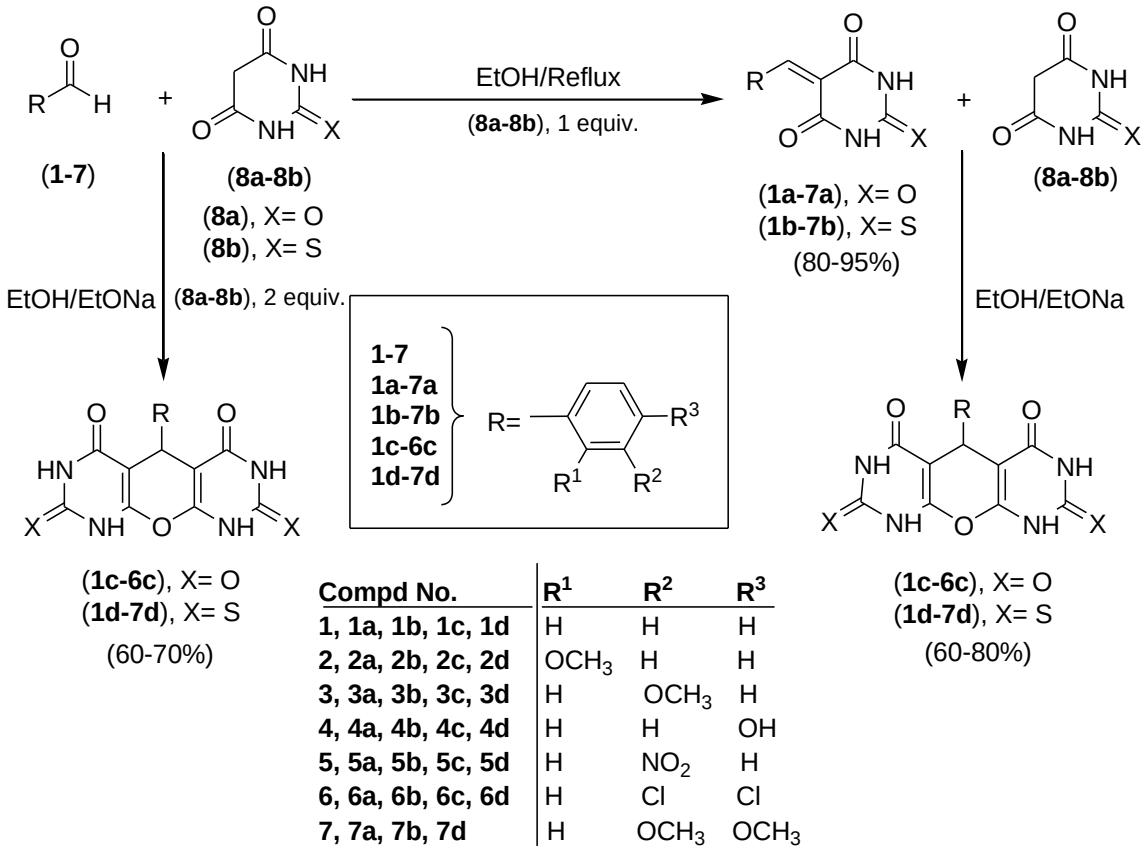
General Procedure for synthesis of Tricyclic heterocycles (**1c-6c**) *and* (**1d-7d**) *from Knoevenagel adducts.*

Knoevenagel adducts **1a-7a** and **1b-7b**, (0.0025 mol. 1 equivalent) were further reacted with barbituric acid/thiobarbituric acid (0.0025 mol. 1 equivalent) were mixed in 20 mL dry ethanol and catalytic amount of sodium ethoxide was added carefully and progress of reaction was monitored through TLC. Upon completion, solvent was removed under vacuum and the crude product was solubilized upon addition of 20 mL of distilled water. The resulting solution was acidified with few drops of diluted HCl till the desired product precipitated out and collected through vacuum filtration followed by several washings with hot ethanol to yield the products (**1c-6c**) and (**1d-7d**) in solid state with good yield (60-80%) (**Scheme 1**).

Spectroscopic Data

9 - phenyl - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8. 4.0.0³, ⁹]tetradeca - 1(10),3(8) - diene - 5,7,11,13 - t etrone. **(1c)**

Yellow solid, yield 60%, m.p. decompose above 365°C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm, *J* Hz) δ 10.19 (4H, s, NH), 7.22 – 7.11 (2H, m, ArH), 7.05 (3H, d, *J* = 6.8, ArH), 5.90 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.95(C), 155.98(C), 151.92(C), 150.62(C), 144.88(C), 129.53(C), 127.47(CH), 126.73(CH), 124.24(CH), 99.53(C), 91.01(C), 30.43(CH). HRMS (EI) calcd for $C_{15}H_{10}N_4O_5$, 326.0651. Found: 326.0657



Compd No.	R^1	R^2	R^3
1, 1a, 1b, 1c, 1d	H	H	H
2, 2a, 2b, 2c, 2d	OCH_3	H	H
3, 3a, 3b, 3c, 3d	H	OCH_3	H
4, 4a, 4b, 4c, 4d	H	H	OH
5, 5a, 5b, 5c, 5d	H	NO_2	H
6, 6a, 6b, 6c, 6d	H	Cl	Cl
7, 7a, 7b, 7d	H	OCH_3	OCH_3

Scheme 1: Synthesis of Tricyclic-heterocycle compounds

9 - (2 - methoxyphenyl) - 2 - oxa - 4,6,12,14 - tetra azatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 5,7,11,13 - tetrone. **(2c)**

Yellow solid, yield 68%, m.p. 305-307 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm, *J* Hz) δ 9.89 (4H, s, NH), 7.09 (2H, dd, *J* = 7.7, 3.8, ArH), 7.04 – 6.93 (1H, m, ArH), 6.73 (1H, dd, *J* = 14.7, 7.5, ArH), 5.85 (1H, s, CH), 3.87 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.87 (C), 161.89(C), 159.45(C), 157.63(C), 151.20(C), 134.58(CH), 132.90(CH), 119.93(CH), 111.44(CH), 91.14(C), 56.36(OCH_3), 27.74(CH). HRMS (EI) calcd for $C_{16}H_{12}N_4O_6$, 356.0757. Found: 356.0748

9 - (3 - methoxyphenyl) - 2 - oxa - 4,6,12,14 - tetra azatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 5,7,11,13 - tetrone. **(3c)**

Brown solid, yield 75 %, m.p. decompose above 320 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm, *J* Hz) δ 10.21 (4H, s, NH), 7.06 (1H, t, *J* = 7.8, ArH), 6.63 (2H, d, *J* = 6.8, ArH), 6.57 (1H, s, ArH), 5.83 (1H, s, CH), 3.64 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.48(C), 164.63(C), 159.33(C), 151.16(C), 146.52(C), 128.78(CH), 119.78(CH), 113.74(CH), 109.47(CH), 91.49(C), 55.16(OCH_3), 30.89(CH). HRMS (EI) calcd for $C_{16}H_{12}N_4O_6$, 356.0757. Found: 356.0762

9 - (4 - hydroxyphenyl) - 2 - oxa - 4,6,12,14 - tetra azatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 5,7,11,13 - tetrone. **(4c)**

Deep yellow solid, yield 69 %, m.p. decompose above 300 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 10.18 (4H, s, NH), 8.88 (1H, s, OH), 6.81 (2H, d, *J* = 8.2, ArH), 6.54 (2H, d, *J* = 8.4, ArH), 5.73 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 166.34(C), 164.56(C), 163.74(C), 154.72(C), 151.13(C), 150.67(C), 127.91(CH), 114.65(CH), 91.78(C), 30.00(CH). HRMS (EI) calcd for $C_{15}H_{10}N_4O_6$, 342.0600. Found: 342.0599

9 - (3 - nitrophenyl) - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene-5,7,11,13 - tetrone. **(5c)**

Brown solid, yield 78 %, m.p. decompose above 350 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 10.32 (4H, s, NH), 7.95 (1H, d, *J* = 6.0, ArH), 7.83 (1H, s, ArH), 7.49-7.44 (2H, m, ArH), 5.99 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 165.86(C), 165.09(C), 151.14(C), 148.05(C), 147.67(C), 134.28(CH), 129.52(CH), 121.49(C), 120.43(CH), 90.88(C), 31.15(CH). HRMS (EI) calcd for $C_{15}H_8N_5O_7$, 371.0502. Found: 371.0516

9 - (3,4 - dichlorophenyl) - 13 - hydroxy - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8),12 - triene - 5,7,11 - trione. **(6c)**

Yellow solid, yield 66 %, m.p. decompose above 300 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 10.21 (4H, s, NH), 7.40 (1H, d, *J* = 8.4, ArH), 7.17 – 7.01 (1H, m, ArH), 6.99 (1H, s, ArH), 5.87 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 165.13(C), 163.34(C), 161.94(C), 151.06(C), 150.59(C), 146.65(C), 129.32(CH), 128.88(CH), 127.79(CH), 90.69(C), 30.80(CH). HRMS (EI) calcd for $C_{15}H_8Cl_2N_4O_5$, 393.9871 Found: 393.9869.

9 - phenyl - 5,13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸] tetradeca - 1(10), 3(8) - diene - 7,11 - dione. **(1d)**

Pink solid, yield 80 %, m.p. decompose above 290 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.73 (2H, s, NH), 11.52 (2H, s, NH), 7.16 (2H, t, *J* = 7.4, ArH), 7.03 (3H, dd, *J* = 17.3, 7.4, ArH), 5.92 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.16(C), 164.39(C), 163.22(C), 143.06(C), 128.09(CH), 127.00(CH), 125.35(CH), 96.33(C), 30.88(CH). HRMS (EI) calcd for $C_{15}H_{10}N_4O_3S_2$, 358.0194. Found: 359.0213.

9 - (2-methoxyphenyl) - 5,13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 7,11 - dione. **(2d)**

Orange solid, yield 74 %, m.p. decompose above 260 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.41 (4H, s, NH), 7.08 – 7.01 (2H, m, ArH), 6.75 (2H, m, ArH), 5.89 (1H, s, CH), 3.59 (3H,s, OCH_3). ^{13}C NMR (75 MHz, DMSO- d_6) δ 172.82(C), 163.75(C), 162.86(C) 157.56(C), 126.79(CH), 121.08(CH), 111.20(CH), 96.29(C), 55.82(OCH_3), 27.66(CH). HRMS (EI) calcd for $C_{16}H_{12}N_4O_4S_2$, 388.0300. Found: 388.0297.

9 - (3-methoxyphenyl) - 5, 13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 7,11 - dione. **(3d)**

Off white solid, yield 69 %, m.p. decompose above 265 °C. ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.76 (2H, s, NH), 11.56 (2H, s, NH), 7.09 (1H, t, *J* = 7.9, ArH), 6.69 – 6.57 (2H, m, ArH), 6.53 (1H, s, ArH), 5.88 (1H, s, CH), 3.65 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.16(C), 164.41(C), 163.28(C), 159.43(C), 144.81(C), 129.07(CH), 122.93(C), 119.64(CH), 113.71(CH), 109.82(CH), 96.30(C), 55.25(OCH_3), 30.89(CH). HRMS (EI) calcd for $C_{16}H_{12}N_4O_4S_2$, 388.0300. Found: 388.0323.

9 - (4 - hydroxyphenyl) - 5,13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 7,11 - dione. **(4d)**

Red solid, yield 77 %, m.p. decompose above 300 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.67 (2H, s, NH), 11.48 (2H, s, NH), 8.97 (1H, s, ArH), 6.78 (2H, d, *J* = 8.2, ArH), 6.55 (2H, d, *J* = 8.5, ArH), 5.80 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.03(C), 164.33(C), 163.13(C), 155.09(C), 132.94(C), 127.84(CH), 114.89(CH), 96.67(C), 30.02(CH). HRMS (EI) calcd for $C_{15}H_{10}N_4O_4S_2$, 374.0143. Found: 374.0139.

9 - (3-nitrophenyl) - 5,13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³,⁸]tetradeca - 1(10),3(8) - diene - 7,11 - dione. **(5d)**

Pink solid, yield 78%, m.p. decompose above 325 °C; ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.81 (2H, s, NH), 11.67 (2H, s, NH), 7.97 (1H, d, *J* = 3.8, ArH), 7.79 (1H, s, ArH), 7.48 (2H, s, ArH), 6.03 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.48(C), 164.11(C), 163.44(C), 148.10(C), 146.04(C), 134.20(CH), 129.79(CH), 121.36(CH), 120.82(CH), 95.58(C), 31.09(CH). HRMS (EI) calcd for $C_{15}H_9N_5O_5S_2$, 403.0045. Found: 403.0053.

9 - (3,4 - dichlorophenyl) - 5,13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³, ⁸] tetradeca - 1(10),3(8) - diene - 7,11 - dione. **(6d)**

Yellow solid, yield 67 %, m.p. decompose above 290 °C. ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.75 (2H, s, NH), 11.69 (2H, s, NH), 7.41 (1H, d, *J* = 8.4, ArH), 7.10 (1H, dd, *J* = 2.1, 1.1, ArH), 7.04 – 6.88 (1H, m, ArH), 5.93 (1H, s, CH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.44(C), 163.92(C), 162.89(C), 145.02(C), 136.63(C), 132.05(CH), 131.75(CH), 127.84(CH), 95.56(C), 30.65(CH). HRMS (EI) calcd for $C_{15}H_8C_{l2}N_4O_3S_2$, 425.9414. Found: 426.9418.

9 - (3,4 - dimethoxyphenyl) - 5,13 - disulfanylidene - 2 - oxa - 4,6,12,14 - tetraazatricyclo[8.4.0.0³, ⁸] tetradeca - 1(10),3(8) - diene - 7,11 - dione. **(7d)**

Brown solid, yield 63 %, m.p. decompose above 320 °C. ^{1}H NMR (300 MHz, DMSO- d_6, δ ppm, *J* Hz) δ 11.64 (2H, s, NH), 11.48 (2H, s, NH), 6.75 (1H, d, *J* = 8.2, ArH), 6.54 (2H, d, *J* = 10.3, ArH), 5.87 (1H, s, CH), 3.67 (3H. s, OCH_3), 3.60 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.10(C), 164.24(C), 163.16(C), 148.58(C), 147.04(C), 135.82(C), 119.32(CH), 111.98(CH), 111.75(CH), 96.56(C), 56.02(OCH_3), 55.98(OCH_3), 30.47(CH). HRMS (EI) calcd for $C_{17}H_{14}N_4O_5S_2$, 418.0406. Found: [M+H_2O] 436.0509

Biological Studies

Antioxidant activity against DPPH

The radical scavenging activity of all synthesised compounds (**1a-7a**, **1b-7b**, **1c-6c**, **1d-7d**) was evaluated employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as described in the reported method [17]. Stock solutions of the compounds were prepared in DMSO at a concentration of 1 mM and working samples (25–200 µM) were prepared from the stock solution by further dilution. 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffered saline (HBS) (60 µL), working sample (10 µL) and DPPH (130 µL, 0.1 mM in methanol) were mixed in a 96-well microplate, resulting in a final concentration of 0.065mM DPPH. DPPH solution in HBS buffer and DMSO without antioxidant compound was considered as negative control and ascorbic acid was used as standard antioxidant [18]. The mixtures were left for 30 min at room temperature in darkness and the absorbance of unreacted DPPH was measured at 520 nm in a SpectraMax microplate reader (Molecular Devices) [19]. The radical scavenging activity was calculated as shown below in (Equation 1).

$$\%\text{age Activity} = \left[\frac{\text{Abs(-ve control)} - \text{Abs(sample)}}{\text{Abs(-ve control)}}\right] \times 100$$

Eq 1

The IC_{50} value of each compound was determined by linear regression method.

Antibacterial studies

The synthesized compounds were tested individually against three bacterial species one gram +ve and two gram -ve; bacterial strains were used in this study, obtained from the American Type Culture Collection (ATCC) and were *Staphylococcus aureus* ATCC 25923 (gram +ve), *Pseudomonas aeruginosa* ATCC 27853 (gram -ve), *Escherichia coli* ATCC 35218 (gram -ve). All the synthesized compounds were dissolved in dimethyl sulfoxide and methanol (50:50) to prepare stock solutions. To check the antibacterial activity of synthesized compounds; Tryptic soy agar (TSA) solution was prepared in distilled water and sterilized. Upon cooling in the inclined test tubes, the desired strain of the bacteria was cultured at 37 °C in the incubator for 12 hours. After growth of bacteria on TSA bacterial colonies were extensively washed with 0.9 % NaCl sterile solution and then further diluted through serial dilution method. From each dilution 1 mL sample mixed with sterile agar in petri dish in triplicate and incubated for 12 hrs and then counted the colony forming units (CFU) by hand with visual observation on LED illuminated table colony counter. After determining the CFU number, bacterial suspensions in saline solution mixed with Tryptic soy broth (TSB) to get 1.053×10^5 CFU/190 µL. In 96-well of microplates 10 µL solution of the synthesized 5-benzylidene pyrimidine derivatives from stock solution along with 190 µL of bacterial suspension to get final concentration of 200 µg/200 µL of compound and 1.00×10^5 CFU/200 µL in three wells along with blank sample (Broth + compound). The antibacterial activity of compounds was compared with the positive control Tetracycline Hydrochloride and negative control containing broth with bacteria and DMSO: Methanol solution. Then 96-Well plate incubated for 12 h at 37 °C and measured the absorbance at 630 nm on SpectraMax M3 Series Microplate Reader repeated experiment in triplicate

and calculated percentage inhibition through formula depicted in (Equation 1).

Docking Studies

Docking studies of synthesized ligand was performed using AutoDock Tools version 1.5.6 (ADT) software. Grid dimension used for all ligands was 90 × 90 × 90 Å separated by 0.375 Å (grid-point spacing). Grid centre coordinate X, Y, and Z were also specified and use centre on macromolecule. Docking runs value was set to the default value of ten (10) for all ligands. Ligand and receptor binding energy/affinity was calculated using ADT software package by search genetic algorithm. Different conformations of ligand were obtained with their respective binding energy/affinity and choose the pose with lowest binding energy as stable one and was employed in the post docking analysis. Ki the inhibition constant was calculated by using the equation (**Equation 2)**.

$$\Delta G = RT \ln Ki \qquad \text{Eq 2}$$

$Ki = e^{(\Delta G/RT)}$ where ΔG is the binding affinity/energy in Kcal/mol, R is gas constant, 1.987 cal/mol/K and T is room temperature, 298.15 K.

ADT results were analysed using Python Molecular Viewer 1.5.6, different binding modes of receptor and ligand were studied in PDBQT format and the binding with lowest binding energy mode were selected. The ligand-receptor interaction with different atom and groups were also observed. The RCSB PDB file 3A47 for isomaltase from *Saccharomyces cerevisiae* was downloaded in PDB format and further processed for docking studies by using BIOVIA Discovery Studio visualizer v16.1.0.15350. Legend structure was optimized using Merck Molecular Force Field (MMFF94s) parameters in Avogadro 1.2.0 [20]. Docking calculations were carried out using AutoDockTools-1.5.6 (ADT) [21]. Non-polar hydrogen atoms were merged after addition of hydrogen atoms. Kollman charges were added to the receptor molecule. Rigid file of receptor molecule was employed for the docking studies. Affinity (grid) maps of 90×90×90 Å grid points and 0.375 Å spacing were generated using the Autogrid program, ADT default parameter and functions were used in the calculation of the electrostatic, bonding and energy calculations. Docking simulations were done using the Lamarckian genetic algorithm (LGA 4.2). Initial position, orientation and all maximum torsions of the ligand molecules ranging from 1-4 were set. The more stable conformation of ligand with receptor along with low Kinase Inhibition (KI uM) value was used to evaluate docking score.

α-Glucosidase Inhibition

For compounds (**1a-6b**) and (**1d-7d**) α-glucosidase inhibition *in-vitro* studies were performed in 96 micro-well plate according to reported method [22], [23] with slight modification; phosphate buffer 100 µL (100 mM) with pH 6.8 was added in each well then 50 µL α-glucosidase 1U/mL commercial solution from *Saccharomyces cerevisiae* was introduced and eventually 10 µL of compound dissolved in DMSO was slowly added and the microplate were incubated at 37°C for 10 min. After which *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) 40 µL (5mM) solution was added and again incubated the reaction mixture at 37°C for further 10 min. The reaction was quenched by 100 µL of a 0.5 M Tris solution and release of p-Nitrophenol was measured at 405 nm SpectraMax spectrometer (Molecular Devices USA). Acarbose was used as standard to compare the activity of synthesized compounds. Each experiment was performed in triplicate and reaction mixture without inhibitor was used as negative control. Percentage of inhibition or activity was calculated using (**Equation 1**). Concentration of 500 mM was employed to screen α-glucosidase inhibition for initial screening. Then the screened compounds with high percentage of inhibition were employed to calculate IC_{50} value using appropriate concentrations.

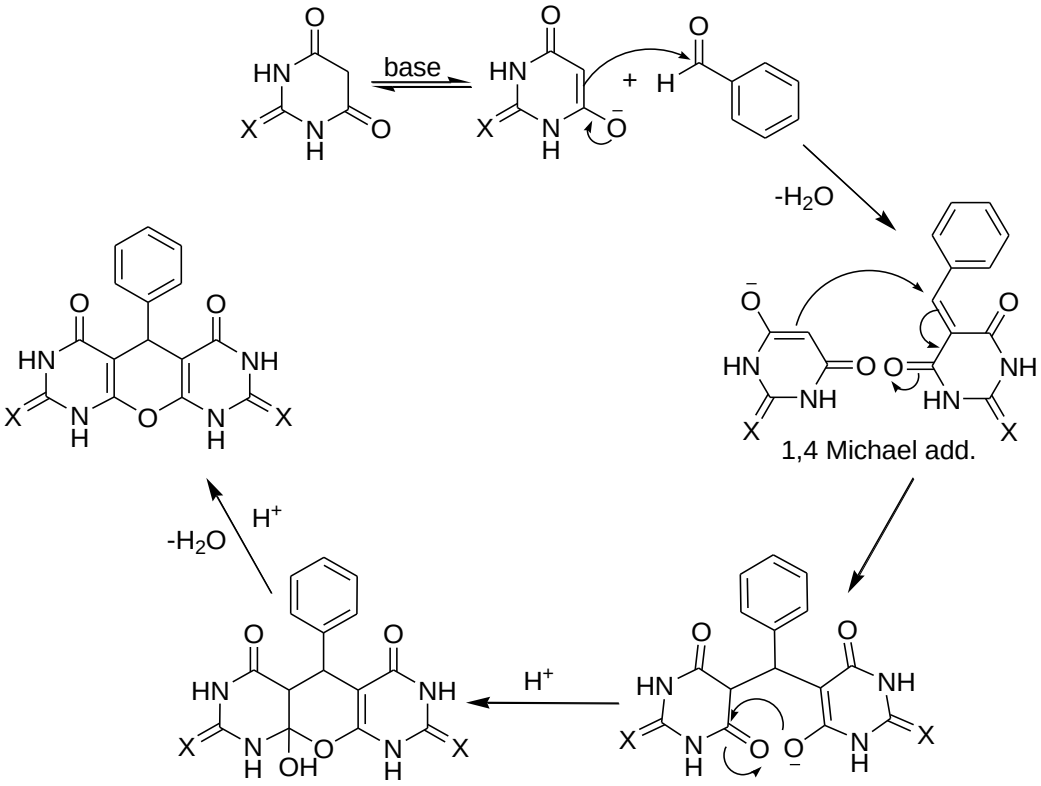
Results and Discussion

Synthesis

Targeted tricyclic compounds (**1c-6c**) and (**1d-7d**) were synthesized by using two different strategies; firstly, single pot multicomponent reaction and the secondly, stepwise (two step) methodology, both led to minimal side reaction with high purity and trifling fuss of purification. In case of multicomponent single pot strategy, one equivalent of aromatic aldehyde and two equivalents of barbituric/thiobarbituric acid was stirred in dry ethanol and catalytic amount of sodium ethoxide was added and the reaction mixture was refluxed for 4-6h which yielded the desired tricyclic heterocyclic compounds in good yields. Whereas, to establish the mechanistic pathway the same reaction was performed *via* stepwise methodology, initially synthesize of 5-benzylidene barbiturate and thiobarbiturate derivatives were availed *via* Knoevenagel reaction [24] and their structure and purity was confirmed through spectroscopic

techniques and eventually the Knoevenagel adducts were further reacted in the presence of sodium ethoxide as catalyst with equimolar amount of barbituric acid/thiobarbituric acid to avail highly functionalized tricyclic heterocycle compounds (**1c-6c**) and (**1d-7d**).

Mechanistic pathway of single pot multicomponent reaction is depicted in the proposed mechanism in Scheme 2; which involved sequential Knoevenagel-Michael reaction followed by dehydration to avail the desired tricyclic heterocycle compounds (**1c-6c**, **1d-7d**). This proposed mechanism was strengthened by the fact that when Knoevenagel adducts were separately isolated and then reacted with equimolar amount of barbituric/thiobarbituric acid in presence of catalytic sodium ethoxide, it yielded the same tricyclic heterocycle compounds (**1c-6c**, **1d-7d**).



Scheme 2: Proposed Mechanism of single pot multicomponent reaction.

All the synthesized compounds (**1c-6c**, **1d-7d**) were extensively characterized by ^{1}H and ^{13}C NMR, mass spectroscopy and elemental analysis. In ^{1}H NMR (**1c-6c**) recorded four NH as a singlet in the range of δ 10.21-9.89 ppm, while methine hydrogen displayed as a singlet at δ 5.73-5.99 ppm and all the aromatic protons substituted at C-4 was also accounted for in the aromatic region. In thiobarbituric acid based tricyclic-heterocycles (**1d-7d**) ^{1}H NMR spectrum of four NH signal split into two separate singlets in the range δ 11.81-11.64 and 11.69-11.48 ppm, while methine hydrogen was observed in the range of δ 6.03-5.80 ppm as a singlet. In compounds (**1c-6c**, **1d-7d**) ^{13}C NMR four quaternary and one methine carbon account for the three cyclic rings; where methine CH was observed in the range of 31.15-27.74 ppm, two C signals accounts for the pyrane ring carbons (C-3, C-15) and (C-12, C-14), recorded between the range δ 96.67-90.88 ppm and δ 155.98-165.09 respectively. Third C signal account for carbonyl carbons (C-2, C-16) show their signals in range of δ 166.48-165.13 ppm, fourth C signal (C-23, C-18) in compounds (**1c-6c**) attributed to carbonyl carbon, while in compound (**1d-7d**) is for thio carbonyl group show signal in the range δ 163.74-151.14 ppm and 173.48-173.03 ppm respectively. Furthermore, all the proposed structures are found consistent and in agreement with the observed HRMS and elemental analysis data.

In-vitro Biological Studies and Molecular Docking

Further biological activity of all synthesized compounds was evaluated through *in-vitro* antibacterial and antioxidant studies to compare activity of precursor compounds (**1a-7a**), (**1b-7b**) with final products (**1c-6c**) and (**1d-7d**). Antibacterial studies were performed at single concentration 200 µM concentration in triplicate repeated two time which reveal that all these compounds have mild bacteriostatic properties (**Figure 1**), though compound **7b** shows excellent inhibition 89.06%.

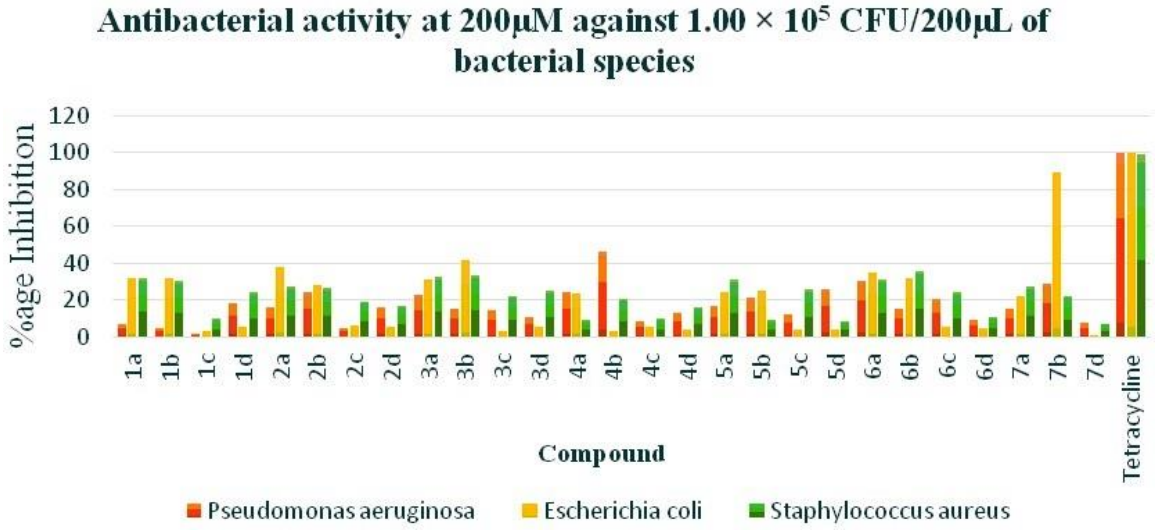


Fig. 1: Percentage of bacterial growth inhibition of synthesized compounds.

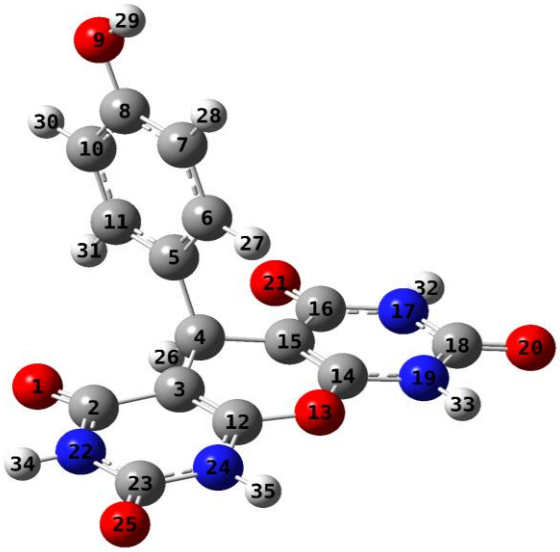


Fig. 2: 3D optimised structure of (4c) GaussView 4.1.2.

In-vitro antioxidant studies were performed against 0.065 mM DPPH radical, which reveal that all compounds possess excellent antioxidant activity against DPPH, while compound (**1c-6c**), (**1d-7d**) have more antioxidant activity than their precursors (**1a-7a**), (**1b-7b**) except few exceptions i.e. **2c, 3c, 5d,** and **7d**. The IC_{50} value of compound **2d, 3d, 4d, 6c** and **6d** show that they have more potent as antioxidant than the standard ascorbic acid.

Tricyclic-heterocycle compounds (**1c-6c**) and (**1d-7d**) were evaluated *in-silico and in-vitro* for α-glucosidase inhibition. *In-silico* evaluation predicted that these compounds have high affinity for α-glucosidase and have ability to penetrate inside the

enzyme to its active sites. Docking score also predicted their low kinase inhibition values (Table-3) and number of binding interactions including hydrogen bonding (Table-5 and representative Fig. 3 and 4) for docked enzyme molecule (PDB ID: 3A47) predicted that these compounds are good candidate for α-glucosidase inhibition. *In-silico* screening was further validated through *in-vitro* experimental technique and result of *in-vitro* α-glucosidase inhibition (Table-4) show that targeted heterocyclic compounds are good α-glucosidase inhibitor IC_{50} value of compound **3c, 3d,** and **5d** are more potent than standard acarbose.

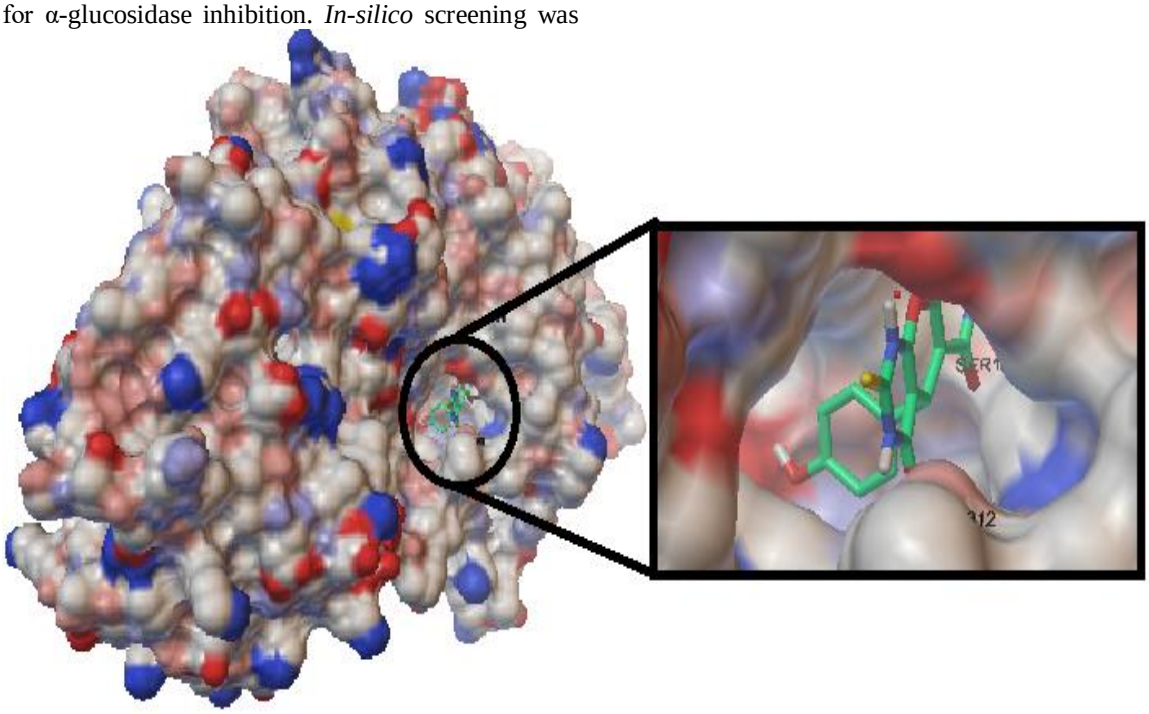


Fig. 3: Docked pose of (4d) inhibitor inside the active cavity of receptor (Protien PDB ID: 3A47) is represented in MSMS style of AutoDock Tools.

Table-1: Antioxidant/DPPH radical scavenging activity of 5-arylidene barbiturates and thiobarbiturates based derivatives

			%age Antioxidant activity against 0.065mM DPPH Conc.				
Compd.	**100µM**	**200µM**	**IC_{50} (µmol/l)**	***Compd.***	**100µM**	**200µM**	**IC_{50} (µmol/l)**
1a	**41.04**	**65.92**	**48.95 ± 0.14**	**4c**	**53.99**	**76.02**	**53.86 ± 4.35**
1b	**36.96**	**74.96**	**47.20 ± 3.5**	**4d**	**42.5**	**62.94**	**22.93 ± 3.73**
1c	**39.19**	**38.62**	**39.19 ± 3.62**	**5a**	**23.91**	**71.65**	**65.65 ± 6.98**
1d	**45.65**	**74.55**	**44.3 ± 2.50**	**5b**	**42.89**	**75.49**	**81.89 ± 2.73**
2a	**38.94**	**68.42**	**83.36 ± 2.79**	**5c**	**55.48**	**71.75**	**58.73 ± 2.76**
2b	**34.98**	**70.67**	**52.39 ± 1.83**	**5d**	**71.18**	**85.75**	**88.68 ± 3.12**
2c	**42.93**	**50.32**	**207.53 ± 0.72**	**6a**	**38.52**	**56.73**	**91.31 ± 1.9**
2d	**44.02**	**71.38**	**31.15 ± 1.51**	**6b**	**55.87**	**71.90**	**67.84 ± 4.02**
3a	**37.90**	**68.43**	**57.17 ± 1.31**	**6c**	**53.20**	**71.53**	**29.23 ± 0.66**
3b	**34.78**	**72.75**	**52.95 ± 2.4**	**6d**	**55.29**	**70.56**	**33.48 ± 1.71**
3c	**58.55**	**75.00**	**58.10 ± 1.64**	**7a**	**49.26**	**62.82**	**42.40 ± 40.2**
3d	**40.99**	**57.69**	**11.47 ± 5.86**	**7b**	**37.39**	**55.5**	**28.54 ± 1.16**
4a	**39.49**	**68.37**	**55.27 ± 0.97**	**7d**	**40.57**	**59.52**	**220.46 ± 4.60**
4b	**34.74**	**60.81**	**185.4 ± 1.84**	***Ascorbic Acid****	**88.20**	**90.44**	**39.33 ± 0.83**

*Positive Control

Table-2: Percentage of bacterial growth inhibition of synthesized compounds.

Compd.	***P.A***	***E.C***	***S.A***	***Compd.***	***P.A***	***E.C***	***S.A***
1a	**06.9**	**31.8**	**31.94**	**4c**	**08.2**	**05.1**	**10.3**
1b	**04.4**	**31.85**	**30.54**	**4d**	**13.0**	**03.8**	**15.7**
1c	**01.7**	**03.1**	**09.7**	**5a**	**17.1**	**24.36**	**31.02**
1d	**18.4**	**05.8**	**24.4**	**5b**	**21.4**	**24.75**	**09.23**
2a	**16.3**	**38.14**	**27.21**	**5c**	**12.1**	**04.4**	**26.2**
2b	**24.3**	**28.32**	**26.7**	**5d**	**25.6**	**04.1**	**08.5**
2c	**04.7**	**06.6**	**19.4**	**6a**	**30.2**	**34.67**	**30.91**
2d	**16.1**	**05.1**	**16.7**	**6b**	**15.1**	**31.78**	**35.5**

3a	**22.7**	**31.24**	**32.74**	**6c**	**20.2**	**05.6**	**24.1**
3b	**15.6**	**41.99**	**33.33**	**6d**	**09.3**	**04.8**	**10.9**
3c	**14.8**	**03.1**	**22.1**	**7a**	**15.3**	**22.19**	**27.31**
3d	**10.6**	**05.4**	**25.1**	**7b**	**28.6**	**89.06**	**22.34**
4a	**24.5**	**23.42**	**8.89**	**7d**	**07.8**	**01.2**	**06.7**
4b	**46.2**	**3.19**	**20.33**	**TC***	**99.9**	**100.0**	**99.0**

* TC= Tetracycline (as positive control), *PA= Pseudomonas aeruginosa*
EC= Escherichia coli. *SA= Staphylococcus aureus*.

Table-3: Docking Score of Tricyclo-hetercyclic Compounds with Receptor molecule PDB code: 3A47 for isomaltase from *Saccharomyces cerevisiae.*

Compd.	Binding Energy Kcal/Mol.	kI uM	Inter-Mol. Energy	Internal Energy	Ligand optimization energy* KJ/mole
1c	**-8.61**	**487.55**	**-8.91**	**-0.72**	**-1001.39**
1d	**-8.27**	**871.35**	**-8.57**	**-0.77**	**-802.14**
2c	**-8.78**	**367.11**	**-9.38**	**-0.96**	**-995.292**
2d	**-8.64**	**467.16**	**-9.23**	**-0.88**	**-795.169**
3c	**-8.97**	**266.97**	**-9.56**	**-0.36**	**-990.026**
3d	**-8.62**	**476.69**	**-9.22**	**-0.74**	**-790.956**
4c	**-8.89**	**304.98**	**-9.49**	**-0.63**	**-1043.15**
4d	**-8.25**	**893.06**	**-8.85**	**-0.65**	**-847.781**
5c	**-9.29**	**153.79**	**-9.89**	**-0.66**	**-867.266**
5d	**-8.44**	**649.67**	**-9.04**	**-0.72**	**-668.677**
6c	**-9.06**	**228.55**	**-9.36**	**-0.79**	**-969.811**
6d	**-9.26**	**163.32**	**-9.56**	**-0.65**	**-771.286**
7d	**-8.47**	**617.3**	**-9.37**	**-0.93**	**-709.698**

*Calculated through Merck Molecular Force Field (MMFF94s) parameters.

Table-4: α-Glucosidase inhibition activity of 5-arylidene barbiturates and thiobarbiturates based derivatives.

Compound	*α-glucosidase inhibition activity %age at 500µM*	IC_{50} (µmol/L)
1c	**43.2**	**-**
2c	**65.14**	**567.53 ± 1.25**
3c	**79.49**	**111.80 ± 3.67**
4c	**35.37**	**-**
5c	**20.16**	**-**
6c	**82.24**	**190.2 ± 8.1**
1d	**75.74**	**297.36 ± 3.6**
2d	**30.1**	**-**
3d	**66.53**	**99.40 ± 1.5**
4d	**16.4**	**-**
5d	**73.9**	**108.75 ± 3.1**
6d	**41.2**	**-**
7d	**83.72**	**138.44 ± 1.45**
Acarbose*	**81.4**	**135.60 ± 4.8**

*Positive Control

Table-5: Ligand receptor interactions predicted through AutoDock.

Compd.No.	Ligand receptor interactions* predicted through AutoDock	Compd. No.	Ligand receptor interactions* predicted through AutoDock
1c	**LYS156, SER157, TYR158, GLY160, SER240, SER241, ASP242, PRO312, LEU313, PHE314, ARG315, ASN415**	**4d**	**LYS156, SER157, TYR158, LEU177, SER241, ASP242, PRO312, ARG315, GLU411**
1d	**LYS156, TYR158, SER157, GLY160, SER240, SER241, ASP242, PRO312, LEU313, PHE314, ARG315, ASN415**	**5c**	**LYS156, TYR158, ASP242, GLN279, HIS280, ASP307, THR310, SER311, LEU313, PHE314, ARG315**
2c	**LYS156, SER157, TYR158, GLY160, SER240, SER241, ASP242, PRO312, LEU313, PHE314, ARG315, ASN415**	**5d**	**LYS156, SER157, TYR158, LEU177, ASP242, SER241, PRO312, PHE314, ARG315, TYR316, ASN415**
2d	**LYS156, SER157, TYR158, GLY160, SER240, SER241, ASP242, PRO312, LEU313, PHE314, ARG315, ASN415**	**6c**	**LYS156, TYR158, SER157, ASP242, GLN279, HIS280, ASP307, THR310, SER311, ARG315,**
3c	**LYS156, SER157, TYR158, GLY160, SER240, SER241, ASP242, HIS280, PHE314, ARG315, ASN415**	**6d**	**TYR158, LEU177, LYS156, ASP242, PRO312, SER241, TYR316, ASN415**
3d	**LYS156, SER157, TYR158, GLY160, SER240, SER241, ASP242, HIS280, PRO312, PHE314, ARG315, ASN415,**	**7d**	**LYS156, SER157, TYR158, LEU177, SER241, ASP242, PRO312, PHE314, ARG315, TYR316, GLU411, ASN415**
4c	**LYS156, SER157, TYR158, GLY160, SER240, SER241, ASP242, PRO312, LEU313, PHE314, ARG315, ASN415,**		

***** Resedues involve in hydrogen bonding with ligand molecules are underlined.

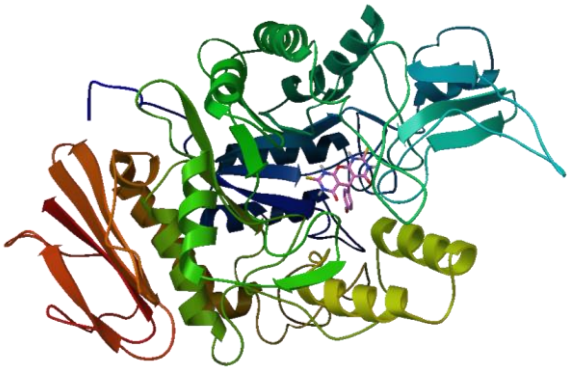


Fig. 4: Superimposition of active sites of glucosidase (Protien PDB ID: 3A47) represented in ribbon on (4d) inhibitor molecule further elaborated in Fig. 4a, 4b.

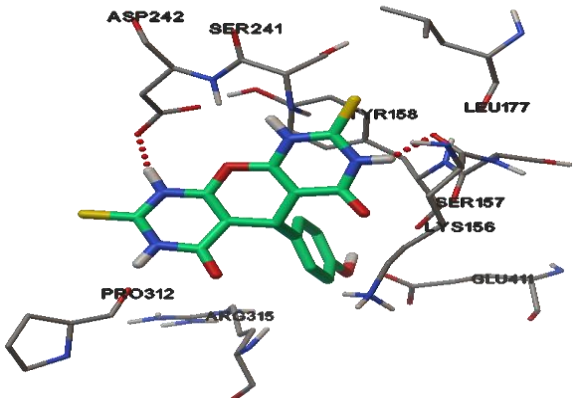


Fig. 4a: Superimposition of site blocked by ligand and hydrogen bonding is represented in dotted line.

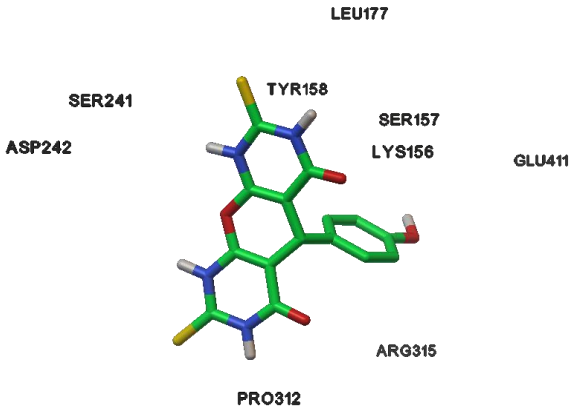


Fig. 4b: Active sites interacting with inhibitor molecule (4d) represented in sticks format.

Conclusion

A new single pot multicomponent methodology is developed which was successfully employed to avail highly functionalized tricyclic-heterocycle compounds (**1c-6c**) and (**1d-7d**) in good yields. The scope of the reaction was extensively studied and mechanism was proposed consisting of sequential Knoevenagel-Michael reaction followed by dehydration to yield desired compounds. All the synthesized tricyclic heterocycle compounds exhibited excellent antioxidant activities comparable to the standard (ascorbic acid) and compounds (**2d, 3d, 4d, 6c** and **6d**) recorded IC_{50} (31.15, 11.47, 22.93, 29.23, 33.48 µmol/L) respectively and were found more potent antioxidant in comparison to standard (39.33 µmol/L). These tricyclic heterocycle compounds were found to be good α-glucosidase inhibitors and IC_{50} value (111.8, 99.4, 108.7 µmol/L) of three compounds (**3c, 3d,** and **5d**) respectively exhibit more robust inhibition as compared to the standard acarbose (135.60 µmol/L). *In-silico* evaluations of α-glucosidase inhibition were also consistent with the *in-vitro* studies and gave a good insight of the structure activity relationship involved in α-glucosidase inhibition.

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Note: The authors declare no competing financial interest.

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