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Synthesis, Characterization and *in vitro* Studies of Some Ethyl 2-Carboxylate-5-monosubstitued 1*H*-indole Derivatives as Potential GSK-3β Inhibitors

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ABSTRACT

The present work focuses on indole derivatives due to their promising inhibition activity toward GSK-3β. New compounds based on the indole moiety were synthesized *via* Japp-Klingemann indole synthesis. The structures of the new compounds were elucidated on the basis of their FTIR, ^{1}H NMR, ^{13}C NMR spectral data, GC-HRMS and elemental analysis. The *in vitro* GSK-3β inhibitory activity of the new compounds was evaluated using a luminance assay technique in terms of IC_{50}. Compound **Aii11** showed excellent inhibitory activity. Compounds **Aii2**, **Aii1** and **Aii3** presented promising GSK-3β inhibitory activity.

KEYWORDS

Indole derivatives, *in vitro*, GSK 3β, Japp-Klingerman.

INTRODUCTION

The multifunctional serine/threonine protein kinase Glycogen synthase kinase-3 (GSK-3) was identified in the late 1970s. GSK-3α and GSK-3β are two isomers of mammalian GSK-3 that share a high degree of similarity in the catalytic region but differ greatly in the N-terminal domain (84% overall and 98% in catalytic domain). Both isomers are found in cells and tissues everywhere and they have similar metabolic properties [1].

GSK-3 plays a critical role in glycogen metabolism, embryogenesis, mitotic regulation, inflammation and neuroplasticity. Inhibition of GSK-3 may provide therapy for several diseases such as cancer, diabetes type-2, chronic inflammatory processes, stroke, bipolar disorders and Alzheimer's disease and so on [2]. Accordingly, searching for GSK-3 inhibitors is an active area in both academic centers and pharmaceutical companies [3].

Particularly, the functional role of GSK-3 in insulin signaling and glucose metabolism makes it a particularly intriguing candidate target for treatment of type 2 diabetes [4]. Activated GSK-3 phosphorylates and thereby inactivates glycogen synthase, an enzyme involved in converting glucose to glycogen for storage. In addition, investigations regarding the relationship between structural differences and the affinity of GSK-3β have been lacking in the literature [5,6]. The present work

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focuses on indole derivatives due to their promising inhibition activity toward GSK-3β.

Indole skeleton is present in the structure of many natural products with high structural complexities and biologically active molecules [7]. For this reason, indole and its derivatives have been used, continuously, in different research areas such as pharmaceuticals, fragrances, agrochemicals, pigments and material science [8,9].

The indole structure is present in the indole-3-carbinol, which is an important antitumor agent. One of the most important indole derivatives is an essential amino acid, tryptophan. It is one of the 22 naturally occurring amino acids. There are many drugs in circulation whose structures contain the indole nucleus, including sumatriptan, a tryptamine [10] derivative used in treatment of migraine headaches, indomethacin and ethodolac [11], which are used as non-steroidal anti-inflammatory drugs and pindolol, a β-adrenoceptor antagonist [12].

Fischer indole synthesis allows the attachment of different substituents at the 2- and 3-positions and on the aromatic ring which by using different substituted ketones, benzene and hydrazine derivatives. The Japp-Klingemann coupling of aryl diazonium salts with β-ketoester or β-ketoacid provides an alternative synthesis of aryl hydrazone derivatives, which are used in Fischer indole synthesis as intermediates [13]. If β-ketoesters are directly treated with aryl diazonium salt, deacylation follows coupling and then indolization occurs to form indole-2-carboxylate ester by Fischer indole mechanism. When β-ketoacid is used, decarboxylation occurs and the final product is 2-acylindole [14].

In this work, an attempt is made to synthesize the promising indole derivatives by Japp-Kingemann reaction. Confirming structures of these compounds by spectral and hyphenated chromatographic techniques. Further *in vitro* luminance assay to evaluate their inhibitory potential. The results demonstrated potential application of these derivatives in treating diabetes mellitus, inflammation and Alzheimer diseases.

EXPERIMENTAL

The laboratory grade reagents were used after purifying and drying [15]. Characterization of the synthesized compounds are mentioned in the individual descriptions, wherein, the starting material used for synthesizing compounds is mentioned, followed by respective melting point (m.p.) mentioned in ºC and recorded in open-end capillaries using Thiele's tube; these are uncorrected. The R_f values mentioned were obtained by performing TLC developed on silica Gel G plates, which were activated at 110 ºC for 0.5 h and *n*-hexane: ethyl acetate:: 2.5:1 v/v with 1 drop of acetic acid as solvent system and iodine vapours were used to localize coloured spots. The purity of the compounds was determined at 254 nm. For further proof of purity of the final compounds, elemental analysis and ^{1}H NMR were determined. ^{1}H & ^{13}C NMR data were measured in DMSO-d_6 as a solvent.

General procedure: All the compounds were synthesized using Japp-Klingemann reaction [16].

Diazotization of *p*-substituted anilines: To a well stirred suspension of *p*-substituted aniline (10 mmol) in 16.6 mL 5 M HCl at 0-5 ºC was added dropwise to a solution of sodium nitrite (1.38 g, 20 mmol, 2 equiv.) in 8 mL water, previously cooled to 0-5 °C in an ice-salt bath. The resulting orange-red mixture was stirred at 0-5 ºC for additional 20 min in an ice-salt bath.

Synthesis of ethyl 2-methyl-3-oxobutanoate anion: Ethyl 2-methyl-3-oxobutanoate (2.512 mL, 1.344 g, 15 mmol) was dissolved in rectified spirit (4.2 mL) and cooled to 0-5 ºC. Then, ice cooled aqueous KOH solution (5.040 g, 90 mmol, 6 equiv. 5 mL in water) previously cooled to 0-5 ºC was dropwise added within *ca.* 0.5 h in order to keep the temperature below 8 ºC. The mixture turned milky which was stirred for further 0.5 h maintaining low temperature.

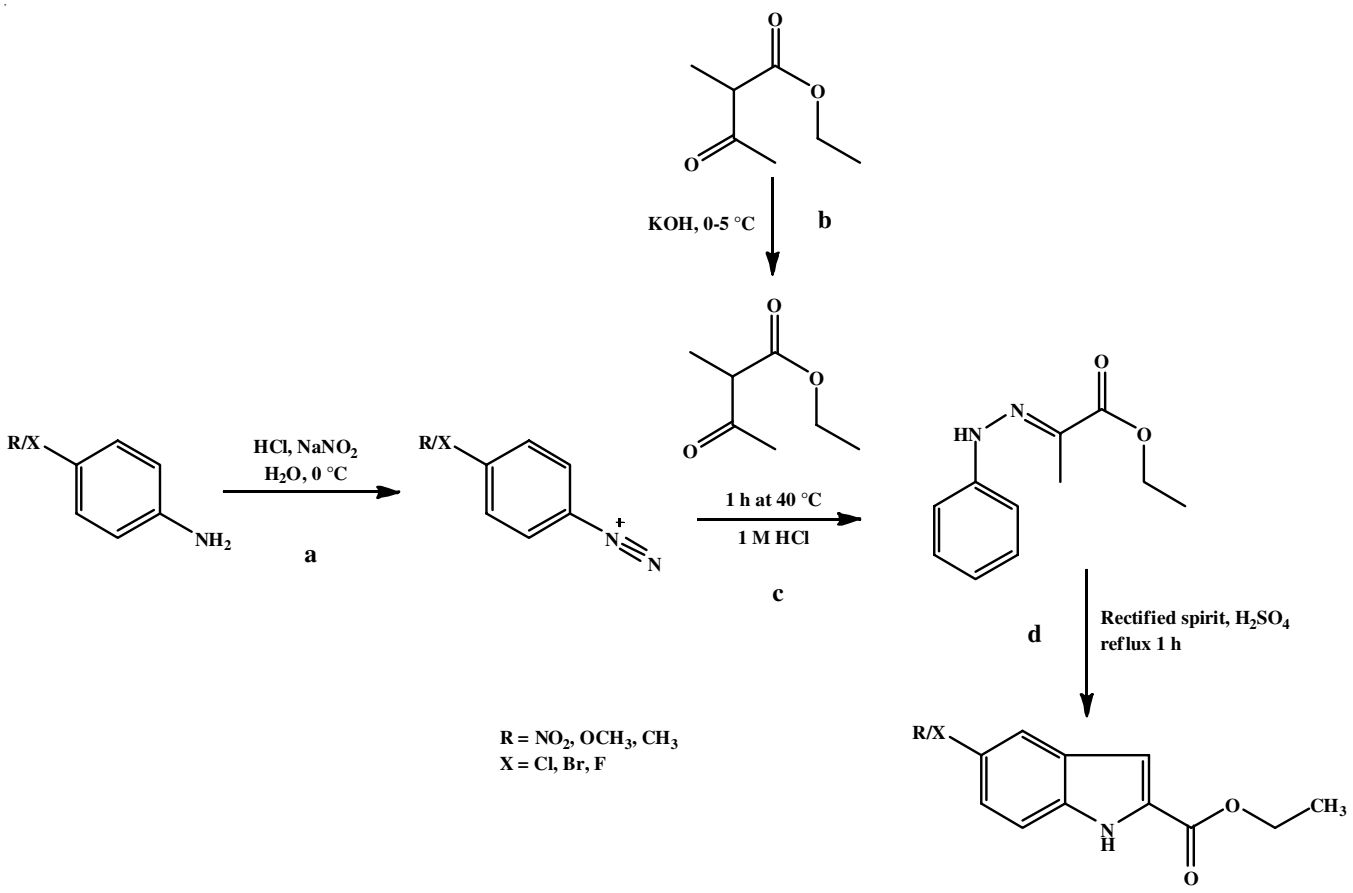
Condensation: Respective hydrazones of diazotized *p*-substituted anilines were obtained by mixing diazonium salts and ethyl 2-methyl-3-oxobutanoate anion.

Ice (50 g) was added to ethyl 2-methyl-3-oxobutanoate anion with stirring at 0-5 ºC in an ice-salt bath, followed by one time addition of diazotized salts and stirring continued for 1 h at 40 ºC. The resultant mixtures were allowed up to room temperature, added 1 M HCl sufficient to maintained pH between 2 to 5. The desired product was extracted with diethyl ether (3 × 50 mL). The combined organic extracts of respective hydrazones were dried over magnesium sulfate, filtered and the filtrate was evaporated to dryness yielding sticky material. These were further used without any purification.

Cyclization: Esters of substituted 1*H*-indole were obtained by cyclization of respective hydrazones (10 mmol), by refluxing hydrazone with conc. H_2SO_4 (2.7 mL, 50.5 mmol, 5.1 equiv.) in rectified spirit (100 mL) for 1 h at 100 ºC. After cooling sufficiently and evaporating the solvent *in vacuo* the obtained residues were treated with 100 mL ice cool water, followed by extraction with dichloromethane (3 × 50 mL), which were dried over magnesium sulfate, filtered and concentrated *in vacuo*. The residues were purified by chromatographic extraction over silica gel column (200-400 mesh) eluting with cyclohexane: ethyl acetate::5:1 resulting white-buff white solids (**Scheme-I**).

Ethyl 5-chloro-1*H*-indole-2-carboxylate (Aii1): 4-Chloroaniline; m.p.: 264-266 ºC; R_f: 0.64; λ_{max} (DMSO): 264. IR (KBr, ν_{max}, cm^{-1}): 3302.24 (N-H *str.*), 3055.35 (C-H Ar. *str.*), 1743.71 (C=O *str.*), 1489.10 (C=C Ar. *str.*), 1172.76 (C-N *str.*). ^{1}H NMR: δ 2.3 (3H, t), 2.5 (2H, q), 3.2-3.7 (1H, broad), 6.9 (1H, s), 7.1 (2H, s). ^{13}C NMR: ($CDCl_3$) δ ppm: 14.47 (C1), 61.12 (C2), 108.76 (C5) 111.94 (C8), 120.84 (C11), 122.66 (C-9), 125.40 (C10), 127.56 (C6), 136.92 (C4), 162.17 (C3). MS (*m/z*): 223 [M]$^+$. CHN Anal.: calcd. (found) %: C, 59.07 (59.00); H, 4.51 (4.50); Cl, 15.85 (15.80); N, 6.26 (6.00); O, 14.31 (14.31).

Ethyl 5-bromo-1*H*-indole-2-carboxylate (Aii2): 4-Bromoaniline; m.p.: 244-246 ºC; R_f: 0.7; λ_{max} (DMSO): 260. IR (KBr, ν_{max}, cm^{-1}): 3320 (N-H *str.*), 2990 (C-H Ar *str.*), 2920 (C-H *str.*), 1690 (C=O *str.*), 1530 (C=C Ar *str.*), 1230 (C-N *str.*), 1200 (C-O *str.*). ^{1}H NMR (δ ppm): 1.35 (3H, t), 4.35 (2H, q), 7.19 (1H, s), 7.23 (1H, s), 7.32 (1H, s).7.75 (1H, s), 8.99 (1H, broad). ^{13}C NMR (δ ppm): 14.45 (C1), 61.36 (C2), 107.88 (C5), 113.42 (C8), 114.03 (C11) 125.01 (C9), 128.39 (C6) 128.67 (C4), 129.17 (C10), 135.37 (C7), 162.17 (C3). MS (*m/z*): [M$^+$] 268; [M+2] CHN Anal.: calcd. (found) %: C, 49.28 (49.21); H, 3.76 (3.50); Br, 29.80 (29.76); N, 5.22 (5.20); O, 11.94 (11.58).



Scheme-I

Ethyl 5-fluoro-1*H*-indole-2-carboxylate (Aii11): 4-Fluroaniline; m.p.: 258-260 ºC; R_f: 0.6; λ_{max} (DMSO): 248; IR (KBr, ν_{max}, cm^{-1}): 3280 (N-H *str.*), 2980 (C-H Ar. *str.*), 2900 (C-H *str.*), 1680 (C=O *str.*), 1520 (C=C Ar. *str.*), 1240 (C-N *str.*), 1200 (C-O *str.*); ^{1}H NMR (δ ppm): 1.34 (3H, t), 4.32 (2H, q), 7.18 (1H, s), 7.25 (1H, s), 7.35 (1H, s), 7.60 (1H, s), 8.98 (1H, broad); ^{13}C NMR (δ ppm): 14.47 (C1), 61.12 (C2), 108.76 (C5), 111.94 (C8), 120.84 (C11), 122.66 (C9), 125.40 (C6) (C4), 127.56 (C10), 136.92 (C7), 162.17 (C3); MS (*m/z*): $[M]^+$ 207.2; CHN Anal. calcd. (found) %: C, 80.17 (80.14); H, 8.07 (8.06); N, 7.48 (7.42); O, 4.27 (4.25).

Ethyl 5-nitro-1*H*-indole-2-carboxylate (Aii5): 4-Nitroaniline; m.p.: 264-266 ºC; R_f: 0.63; λ_{max} (DMSO): 279; IR (KBr, ν_{max}, cm^{-1}): 3310 (N-H *str.*), 2990 (C-H Ar. *str.*), 2920 (C-H *str.*), 1690 (C=O *str.*), 1530 (C=C Ar. *str.*), 1330 (C-N *str.*), 1235 (C-O *str.*); ^{1}H NMR (δ ppm): 1.55 (3H, t), 4.45 (2H, q), 7.39 (1H, s), 7.50 (1H, s), 8.21 (1H, s), 8.69 (1H, s), 9.40 (1H, broad); ^{13}C NMR (δ ppm): 14.42 (C1), 61.81 (C2), 110.45 (C5) 112.19 (C8), 120.05 (C11), 120.54 (C9), 126.78 (C4), 130.81 (C6) 139.25 (C7), 142.75 (C10), 161.26 (C3); MS (*m/z*): $[M]^+$. 234.21; Anal. calcd. (found) %: C, 56.41 (56.12); H, 4.30 (4.27); N, 11.96 (12.02); O, 27.33 (27.42).

Ethyl 5-methoxy-1*H*-indole-2-carboxylate (Aii6): m.p.: 258-260 ºC; R_f: 0.69; λ_{max} (DMSO):256; IR (KBr, ν_{max}, cm^{-1}): 3310 (N-H *str.*), 3000 (C-H *Ar. str.*), 2960 (C-H *str.*), 1700 (C=O *str*), 1540 (C=C Ar. *str.*), 1320 (C-N *str.*), 1260 (C-O *str.*); ^{1}H NMR (δ ppm): 1.55 (3H, t), 4.45 (2H, q), 7.39 (1H, s), 7.50 (1H, s), 8.21 (1H, s), 8.69 (1H, s), 9.40 (1H, broad); ^{13}C NMR ($CDCl_3$, δ ppm): 14.42 (C-1), 61.81 (C2), 110.45 (C5) 112.19 (C8), 120.05 (C11), 120.54 (C9), 126.78 (C4), 130.81 (C6) 139.25 (C7), 142 (C10),) 162.17 (C3); MS (*m/z*): $[M]^+$ 234.21; CHN Anal.: calcd. (found) %: C, 80.17 (80.14); H, 8.07 (8.06); N, 7.48 (7.44); O, 4.27 (4.23).

Ethyl 5-methyl-1*H*-indole-2-carboxylate (Aii3): m.p.: 264-266 ºC; R_f: 0.53; λ_{max} (DMSO): 253; IR (KBr, ν_{max}, cm^{-1}): 3302.24 (N-H *str.*), 3055.35 (C-H Ar. *str.*), 1743.71 (C=O *str.*), 1489.10 (C=C Ar. *str.*), 1172.76 (C-N *str.*); ^{1}H NMR (δ ppm): 1.55 (3H, t), 4.45 (2H, q), 7.39 (1H, s), 7.50 (1H, s), 8.21 (1H, s), 8.69 (1H, s), 9.40 (1H, broad); ^{13}C NMR ($CDCl_3$, δ ppm): 14.42 (C-1), 61.81 (C2), 110.45 (C5) 112.19 (C8), 120.05 (C11), 120.54 (C9), 126.78 (C4), 130.81 (C6) 139.25 (C7), 142 (C10), 162.17 (C3); MS (*m/z*): $[M]^+$ 203; CHN Anal.: calcd. (found) %: C, 70.92 (70.40); H, 6.45 (6.52), N. 6.89 (6.78); O, 15.74 (15.55).

***In vitro* study:** The GSK3β Assay Kit was purchased and procured from BPS Bioscience, San Diego, USA. The GSK3β Assay Kit is designed to measure GSK3β activity for screening and profiling applications using Kinase-Glo® (Promega) as a detection reagent. The GSK3β Assay Kit comes in a convenient 96-well format, with enough purified recombinant GSK3β enzyme, GSK3β substrate (GSK substrate peptide), ATP and kinase assay buffer for 100 enzyme reactions.

Procedure for *in vitro* assay: GSK3β inhibitory activity was evaluated using the method developed by Gameiro *et al.* [17] with modifications. Compounds were dissolved (0.1 µM to 10 µM) in assay buffer, containing 40 mM Tris (pH 7.5), 20

mM $MgCl_2$, 0.1 mg/mL BSA (bovine serum albumin) and 50 µM dithiothreitol (DTT) at the desired concentrations. Firstly, 10 µL of enzyme (10 ng) and 10 µL of each compound were mixed for 30 min. Then, 20 µL of mixture of ATP (1 µM) and GSK3β peptidic substrate were added to each well. The mixture was incubated for 1 h at 30 ºC. Thereafter, the remaining ATP concentration was measured with the Kinase-Glo system following instructions from the supplier. Luminescence was measured in an Orion II microplate luminometer (Berthold, Germany) as relative light units (RLU). GSK3β activity is proportional to the difference between total ATP and remaining ATP after the enzymatic reaction, activity was considered to be 100% in the absence of an inhibitor. The IC_{50} values were calculated by non-linear regression analysis of individual concentration-response curves using GraphPad Prism 5.0 software (San Diego, USA) [18].

RESULTS AND DISCUSSION

Compounds **Aii1**, **Aii2**, **Aii11**, **Aii5**, **Aii6**, **Aii3** were synthesized using Japp-Klingemann reaction. All synthesized derivatives were characterized by IR, ^{1}H NMR, ^{13}C NMR and GC-HRMS. Chromatogram obtained by gas chromatography shows single peak with retention time in range of 23-30 min for all the synthesized compounds. This indicates purity of compounds.

The ^{1}H NMR spectra of synthesized indole derivatives in general, reflect the presence of three methyl protons, two methylene protons and one secondary amino proton. The signal around 7.1 δ ppm apparent in all ^{1}H NMR spectra is due to a proton-containing standard impurity, $CHCl_3$, in the solvent used for the purpose $CDCl_3$. The ^{1}H NMR spectra of all the synthesized compounds warrant the methyl group (t, 1.35 δ ppm) being placed adjacent to the methylene group (q, 4.33-4.45 δ ppm). The multiplet appearing at 7.2-7.8 δ ppm represents aromatic protons and the substitution at 5H position of indole nucleus. The H-bonded to nitrogen nucleus undergo an intermediate rate of exchange, where the NH proton is partially decoupled. This is indicated by broad NH signal results at (8.9-9.40 δ ppm).

The ^{13}C NMR spectra of these compounds reflect the presence of eleven carbon atoms; one carbonyl, eight aromatic and two aliphatic. The mass spectra reflects the molecular weight of **Aii1**, **Aii2**, **Aii11**, **Aii5**, **Aii6**, **Aii3**. Characteristic isotopic peak is obtained in mass spectra of **Aii1** and **Aii2** due to presence of chloro and bromo substitution at 5th position.

The synthesized compounds were subjected to *in vitro* assay of GSK3β inhibition. The obtained results suggest that the compounds did interacted with the enzyme in its ligand binding site. The IC_{50} values calculated for the compounds is tabulated in Table-1. A comparative concentration response curve of all the compounds is illustrated in Fig. 1.

Conclusion

In present study, the synthesis of ethyl 2-carboxylate-5-monosubstitued 1*H*-indole derivatives using Japp-Klingemann

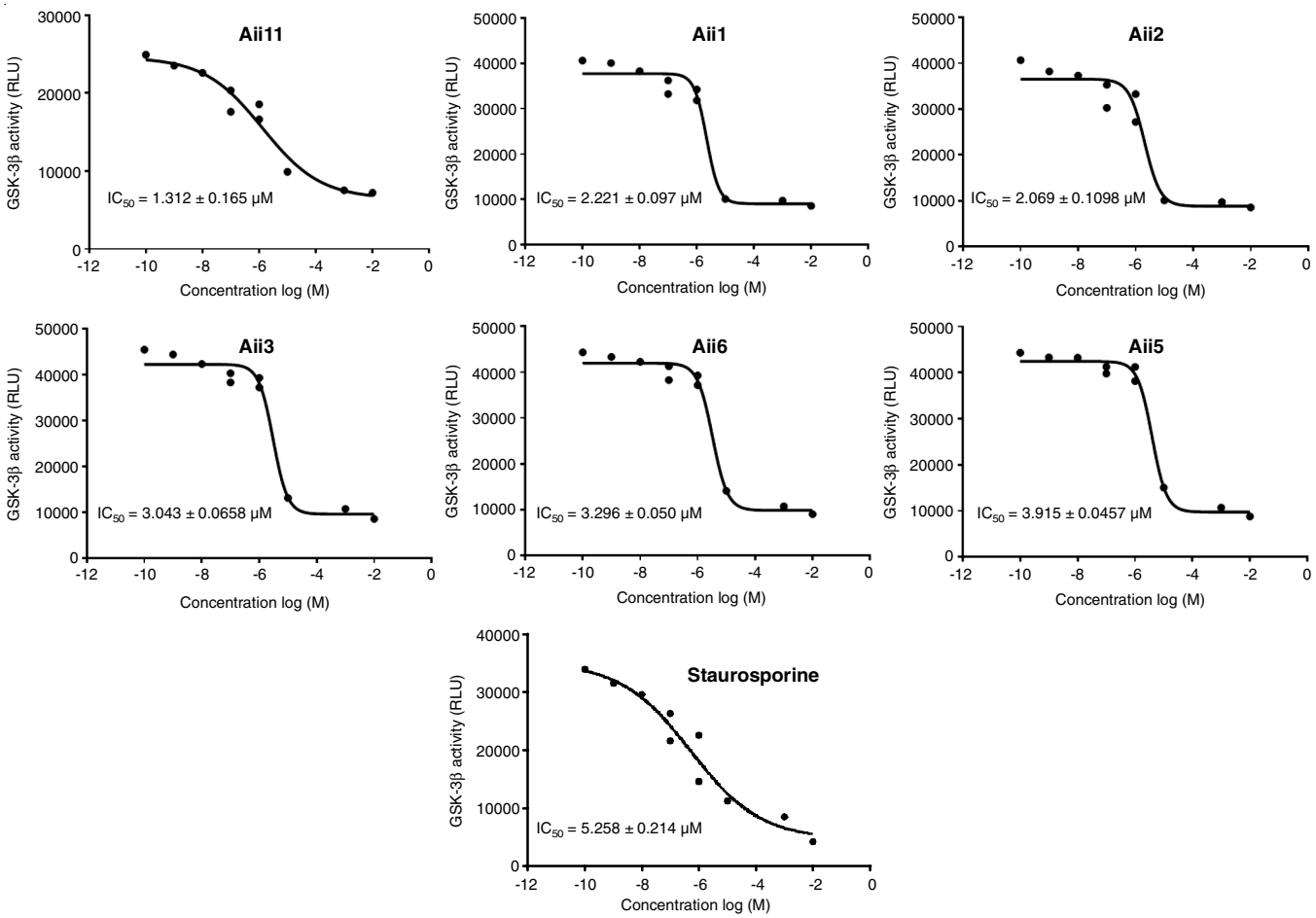


Fig. 1. Concentration response curve of all the compounds

TABLE-1
IC_{50} VALUES OF COMPOUNDS AND THE STANDARD DRUG (STAUROSPORINE)

Compound	GSK3β enzyme assay
Aii11	1.312 ± 0.165 µM
Aii2	2.069 ± 0.1098 µM
Aii1	2.221 ± 0.097 µM
Aii3	3.043 ± 0.0658 µM
Aii6	3.296 ± 0.050 µM
Aii5	3.915 ± 0.0457 µM
Staurosporine	5.258 ± 0.214 nM

reaction was performed. The synthesized molecule were tested for *in vitro* GSK3β enzyme inhibition assay. All the screened compounds exhibited potent GSK3β enzyme inhibition activity. The standard drug staurosporine, exhibited 5.258 ± 0.214 nM IC_{50} value. Compounds **Aii11**, **Aii2**, **Aii1**, **Aii3**, **Aii6** and **Aii5** displayed 1.312 ± 0.165, 2.069 ± 0.1098, 2.221 ± 0.097, 3.043 ± 0.0658, 3.296 ± 0.050 and 3.915 ± 0.0457 µM IC_{50} values, respectively. Compound **Aii11** was found to have most inhibitory potential, which can be treated as lead candidate for the development novel GSK3β enzyme inhibitors for the treatment of diabetes.

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