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In vitro Cytotoxic Activity against MCF-7 Breast Cancer Cells Line: Pregabalin and Chiral Amino Acid-based Peptides

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Because of the medicinal application of peptides in the current drug discovery process, we report the synthesis and *in vitro* anticancer activity of various pregabalin containing peptides using chiral amino acids. The structures of newly synthesized compounds were assigned based on IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis. Synthesized peptides were screened for their anticancer activity on human breast cancer MCF-7 cells line by MMT assays method. Evaluation of anticancer activity shown that compound **13a** (16.65% cell proliferation) was found to be most active against selected MCF-7 cell line.

Keywords: Peptide synthesis, Pregabalin, Cytotoxicity, Breast cancer, MCF-7 cell line.

INTRODUCTION

Cancer is a highly threatening disease to humans and is the major cause of death globally. Therefore, developing and synthesizing highly efficient anticancer agents to treat cancer are the main goals of the drug specialists and scientific community [1-3]. Among various types of cancers, breast cancer, accounting for 23% and 14% of the total cancer cases and cancer deaths, respectively, is the most commonly diagnosed cancer and is the leading cause of cancer related deaths in females. Breast cancer does not indicate a single disease but many molecularly distinct tumours that originate from epithelial cells of the breast [4,5]. MCF-7 is the commonly used cell line of breast cancer and is propagated for numerous years. It is a suitable model cell line for investigations, such as drug related studies, on breast cancer [6,7].

Chemotherapeutic agents acquired from taxanes, vinca alkaloids and anti-tubulins cause peripheral neuropathy, against which no validated treatment is available [8]. A γ-aminobutyric acid analogue, pregabalin (brand name: Lyrica), is used to treat neuropathic pain, epilepsy, restless leg syndrome, fibromyalgia, and generalized anxiety disorder. Case reports and studies have indicated that pregabalin is effective against neuropathic pain [9,10]. Moreover, cancer-related neuropathic pain negatively affects patients' life quality and is associated with delays in the ongoing cancer treatment and dose reduction [11]. Novel cancer treatment strategies based on bioactive peptides are effective. Naturally occurring and artificially prepared peptides are extensively investigated [12]. Numerous promising peptides are successfully used to treat various types of cancer [13,14]. Anticancer peptides (ACPs) exhibit several excellent properties, including reduced side effects, high specificity and desired tumour penetration. Therefore, ACPs are considered potential candidates for cancer treatment [15-18]. On the basis of the current developments made with novel peptides for treating patients with cancer and pregabalin efficacy, this study screened the antitumour effects of chiral amino acid based peptides and pregabalin (**13a-j**) against a selected cell line of MCF-7 (breast cancer).

EXPERIMENTAL

All the chemicals and solvents required for the synthesis were purchased from JSK Fine, Avra Synthesis, Finar and Spectrochem. The majority of the reactions were carried out by standard procedure for the exclusion of moisture. The composition (%) of element (CHN) for the synthesized compounds were determined by using Perkin-Elmer 2400 CHN analyzer. Melting points were determined with the help of Optimelt MPA100, an automated apparatus. IR spectra of all compounds were recorded on Shimadzu FTIR-8400 spectrophotometer in ATR. ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) were obtained using Bruker AVANCE II Spectrometer where DMSO-d_6 was used as solvent and TMS as an internal reference. Mass spectra analysis was conducted on Jeol-JMSD 300 mass spectrometer at 70 eV. TLC plates used for monitoring the completion of the reaction were purchased for Merck (TLC silica gel 60 F_{254}). Visualization was made under UV light (254 and 365 nm) or with iodine vapour.

Synthesis of 3-(((*tert*-butoxycarbonyl)amino)methyl)-5-methylhexanoic acid (2): In 250 mL round bottom flask, pregabalin (10 g, 0.62 mol) and NaOH (2.7 g, 0.65 mol) solution dissolved in water (50 mL) was added along with slowly and dropwise addition of Boc anhydride (15.2 g, 0.69 mol) at 0-5 ºC. It was stirred at room temperature overnight (12 h). The progress of the reaction was monitored by TLC using dichloromethane:methanol (9:1) as a mobile phase (R_f: 0.2). After completing the reaction, the 3-4 pH was maintained using diluted 30 mL hydrochloric acid [6:4, HCl:H_2O] at below 5 ºC. The reaction mixture was filtered under vacuum and dried in a vacuum dryer. It was found as white crystalline powder with 90% of yield.

Synthesis of 2-((*tert*-butoxycarbonyl)amino)propanoic acid (4): In 250 mL round bottom flask, L-alanine (10 g, 1.12 mol) and NaOH pellets (5.4 g, 1.35 mol) were dissolved in water (50 mL). In these solution, dropwise added of Boc anhydride (29.3 g, 1.35 mol) at 0-5 °C temperature. It was stirred at room temperature overnight. The progress of the reaction was monitored by TLC using dichloromethane:methanol (9:1) as a mobile phase (R_f: 0.3). After completing the reaction, the pH (3-4) was maintained by dropwise addition of 30 mL diluted hydrochloric acid [6:4, HCl:H_2O] at below 5 ºC. The separated solid was filtered off under vacuum and was dried in a vacuum dryer at 55 °C temperature. It was found as white crystalline powder with 90% of yield.

Synthesis of methyl-2-amino-3-phenylpropanoate hydrochloride (6): To a solution of L-phenylalanine (**5**) (10 g, 0.60 mol) in methanol (50 mL), $SOCl_2$ (7.2 g, 0.90 mol) was added in a dropwise manner by maintaining the temperature below 0 ºC. After addition, it was stirred at room temperature for 12 h and was monitored by TLC with mobile phase CH_2Cl_2:CH_3OH (8:2). It was distilled off under reduced pressure and washed hexane with vigorous stirring to afford the product **6**. It was used instantly in the next step without further purification.

Synthesis of methyl-2-(3-(((*tert*-butoxycarbonyl)amino)methyl)-5-methylhexanamido)-3-phenylpropanoate (7): Intermediate **2** (3.0 g, 0.11 mol) was dissolved in DMF (30 mL) and carried out temperature below 5 ºC. To this reaction mixture, $C_2H_4Cl_2$ (2.53 g, 0.13 mol) and 4-dimethylaminopyridine (4.5 g, 0.36 mol) were added by maintaining the temperature below 5 °C and stirred well for 10 min. It was fed by previously prepared intermediate **6** (2.39 g, 0.11 mol) and again stirred overnight at room temperature. After the completion of reaction monitored on TLC using CH_2Cl_2:CH_3OH (7:3) as mobile phase, the product was poured into cold water and stirred vigorously for 1 h. Extract the product with MDC (50 mL × 3). The organic layer was washed with brine followed by dried using sodium sulfate and distilled off to get the oily mass of product **7** with 86% yield.

Synthesis of methyl-2-(3-(aminomethyl)-5-methylhexanamido)-3-phenylpropanoate hydrochloride salt (8): In a 250 mL conical flask, a solution of compound **7** (3.8 g, 0.87 mol) was dissolved in dioxane (30 mL) and cooled to 0 ºC. After sometimes the reaction mass appeared clear and was purged by HCl gas (1.0 h). The reaction mixture was stirred for 3-4 h at room temperature. The reaction was monitored by TLC using mobile phase CH_2Cl_2:CH_3OH (9:1 mL). It was distilled off under reduced pressure and washed twice with hexane with vigorous stirring to afford the product **8** and found as white hygroscopic solid material.

Synthesis of methyl-(3-((-2-((*tert*-butoxycarbonyl)-amino)propanamido)methyl)-5-methyl hexanoyl)-L-phenylalaninate (9): Compound **4** (1.48 g, 0.78 mol) was dissolved in DMF (15 mL) and carried out temperature below 5 ºC. To this reaction mixture, $C_2H_4Cl_2$ (1.80 g, 0.94 mol) and 4-dimethylaminopyridine (2.87 g, 2.35 mol) were added by maintaining the temperature below 5 ºC and stirred well for 10 min. It was fed by previously prepared intermediate **8** (2. 9 g, 0.78 mol) and was stirred overnight at room temperature. After completion of reaction monitored on TLC using CH_2Cl_2:CH_3OH (7:3) as mobile phase, the product was poured into cold water and stirred vigorously for 1 h. Extract the product with CH_2Cl_2 (35 mL × 3). The organic layer was washed with brine followed by dried using sodium sulfate and distilled off to get the yellowish oily mass of product **9** with 83% yield.

Synthesis of (3-((-2-((*tert*-butoxycarbonyl)amino)propanamido)methyl)-5-methylhexanoyl)-L-phenylalaninate hydrochloride salt (10): A solution of compound **9** (3.4 g, 0.67 mol) was dissolved in dioxane (30 mL) and cooled to 0 ºC. After sometimes the reaction mass appeared clear and was purged by HCl gas (1.0 h). The reaction mixture was stirred for 3-4 h at room temperature. The reaction was monitored by TLC using mobile phase CH_2Cl_2:CH_3OH (9:1 mL). It was distilled off under reduced pressure and washed twice with hexane with vigorous stirring to afford the product **10** and found as white solid material.

Synthesis of methyl-(5-methyl-3-((-2-(substituted arylamino)propanamido)methyl)hexanoyl)-L-phenylalaninate (11a-d): In round bottom flask (moisture-free) containing aromatic acid (0.23 mol) dissolved in DMF (10 mL) was charged with $C_2H_4Cl_2$ (0.35 mmol) and 4-dimethylaminopyridine (0.73 mol) at below 5 ºC. It was stirred for 10 min at room temperature and added compound **10** (0.23 mol) portionwise. The

reaction mass was stirred at room temperature overnight and was monitored by TLC using dichloromethane:methanol (8:2 mL) mobile phase system. After completion of the reaction, the reaction mass was poured into crushed ice and stirred vigorously for 1 h. Extract the product with CH_2Cl_2 (50 mL × 3). The organic layer was washed with brine followed by dried using sodium sulfate and concentrated under reduced pressure to get the oily mass of product **11a-d** with 70% yield.

Synthesis of (5-methyl-3-((-2-(substituted phenylamino)-propanamido)methyl)hexanoyl)-L-phenylalanine (12a-d): For the hydrolysis of ester, compound **11a-d** (0.21 mol) was dissolved in the binary solvent system [water:THF (1:2)] (12 mL). To this, LiOH (0.32 mol) was added and stirred at room temperature overnight. The progress of the reaction was carried out by TLC. After completing the reaction, it was extracted thrice with 50 mL ethyl acetate and treated with an aqueous layer with 2 N HCl (20 mL) to adjust pH 2. It was further extracted thrice with 50 mL CH_2Cl_2. The organic layer was washed with brine followed by dried using sodium sulfate and concentrated under reduced pressure to get the product **12a-d** with 81% yield.

Synthesis of 3-((-2-(substituted arylamino)propanamido)methyl)-5-methyl-*N*-(-1-oxo-1-substitueted aryl-3-phenylpropan-2-yl)hexanamide (13a-j): For the final product synthesis, compound **12a-d** (0.1 mol) dissolved in DMF (5 mL) was added in *N*,*N*-diisopropylethylamine (3 mmol). It was charged with $C_2H_4Cl_2$ (0.15 mmol) and 4-dimethylaminopyridine (0.48 mol) at below 5 ºC. It was stirred for 10 min at room temperature and added various aromatic amines (0.11 mol) portionwise. The reaction mass was stirred at room temperature overnight and monitored by TLC using CH_2Cl_2:CH_3OH (8:2) mobile phase system. The reaction mass was poured into crushed ice and stirred vigorously for 1 h. Extract the product thrice with 50 mL CH_2Cl_2. The organic layer was washed with brine followed by dried using sodium sulfate and concentrated under reduced pressure to final product **13a-j**. The crude material was purified by column purification using CH_2Cl_2 and CH_3OH mobile phase using silica 60-120 as stationary phase.

***N*-(1-((4-Methyl-2-(2-oxo-2-((1-oxo-3-phenyl-1-((3-(trifluoromethyl)phenyl)amino)propan-2-yl)amino)ethyl)-pentyl)amino)-1-oxopropan-2-yl)nicotinamide (13a):** Yield:65%, m.p.: 260 ºC, white solid. IR spectrum (ATR), ν_{max}, cm^{-1}: 3682 [ν(NH) *sec*. amine], 2980 [ν(CH) arom. ring], 1639 [ν(CO) amide carbonyl], 1446 [ν(C=C) arom. ring], 1332 [ν(CN) C-N linkage], 1028 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.679-0.754 (m, 6H), 0.773-0.899 (m, 2H), 0.932-1.016 (m, 1H), 1.197-1.240 (m, 2H), 1.467-1.362 (m, 1H), 1.817-1.995 (m, 2H), 2.071-2.119 (m, 1H), 2.822-2.953 (m, 2H), 3.048-3.128 (m, 2H), 4.430-4.467 (m, 1H), 4.404-4.686 (m, 1H), 7.173-7.212 (m, 1H), 7.230-7.325 (m, 4H), 7.398-7.418 (m, 1H, *J* = 8 Hz), 7.474-7.564 (m, 2H), 7.778-7.799 (d, 1H, *J* = 8.4 Hz), 7.873-7.903 (t, 1H, *J* = 6 Hz), 8.074 (s, 1H), 8.203-8.233 (dt, 1H, *J* = 8 Hz), 8.334-8.353 (d, 1H, *J* = 7.6 Hz), 8.688-8.705 (dd, 2H, *J* = 4.8 Hz), 9.041-9.045 (d, 1H, *J* = 1.6 Hz), 10.453 (s, 1H). ^{13}C NMR spectrum, δ, ppm: 22.15, 27.23, 29.17, 32.69, 37.65, 40.95, 48.36, 53.98, 57.03, 63.59, 72.69, 120.36, 125.85, 126.69, 128.54, 130.50, 131.68, 133.24, 133.24, 133.89, 134.82, 134.82, 136.69, 138.36, 140.39, 143.54, 145.47, 153.98, 157.35, 168.20, 171.58, 174.36, 176.58. Mass spectrum, *m/z*: 625.29 $[M]^+$. Anal. calcd. (found) % for $C_{33}H_{38}N_5O_4F_3$: C, 63.35 (63.38); H, 6.12 (6.08); N, 11.19 (11.15); O, 10.23 (10.28).

***N*-(1-((4-Methyl-2-(2-oxo-2-((1-oxo-3-phenyl-1-(pyridin-2-ylamino)propan-2-yl)amino)ethyl)pentyl)amino)-1-oxopropan-2-yl)nicotinamide (13b):** Yield: 56%, m.p.: 272 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3635 [ν(NH) *sec*. amine], 2956 [ν(CH) arom. ring], 1631 [ν(CO) amide carbonyl], 1477 [ν(C=C) arom. ring], 1365 [ν(CN) C-N linkage]. ^{1}H NMR spectrum, δ, ppm: 0.690-0.767 (m, 6H), 0.786-0.857 (m, 1H), 0.942-0.993 (m, 1H), 1.123-1.128 (m, 1H), 1.304-1.364 (m, 3H), 1.507-1.574 (m, 1H), 1.847-1.958 (m, 2H), 2.028-2.078 (m, 1H), 3.031-3.093 (m, 1H), 3.112-3.176 (m, 1H), 3.585-3.660 (m, 1H), 4.415-4.487 (m, 1H), 4880-1.938 (m, 1H), 7.171-7.275 (m, 9H), 7.487-7.520 (m, 1H), 7.866-7.896 (m, 1H), 8.215-8.295 (m, 3H), 8.699-8.720 (m, 2H), 9.048-9.052 (d, 1H, *J* = 1.6 Hz). ^{13}C NMR spectrum, δ, ppm: 21.21, 25.21, 25.21, 27.02, 35.95, 39.35, 44.95, 46.68, 51.98, 52.69, 68.86, 118.65, 121.48, 124.56, 128.90, 132.36, 132.36, 133.06, 133.06, 139.02, 141.68, 146.39, 148.38, 150.68, 153.18, 156.28, 157.64, 164.65, 170.09, 173.28, 175.34. Mass spectrum, *m/z*: 558.30 $[M]^+$. Anal. calcd. (found) % for $C_{31}H_{38}N_6O_4$: C, 66.65 (66.71); H, 6.86 (6.89); N, 15.04 (15.00); O, 11.46 (11.39).

***N*-(1-((4-Methyl-2-(2-oxo-2-((1-oxo-3-phenyl-1-((3-(trifluoromethyl)phenyl)amino)propan-2-yl)amino)ethyl)-pentyl)amino)-1-oxopropan-2-yl)-4-(trifluoromethyl)-benzamide (13c):** Yield: 56%, m.p.: 272 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3624 [ν(NH) *sec*. amine], 3209 [ν(CH) arom. ring], 1691 [ν(CO) amide carbonyl], 1512 [ν(C=C) arom. ring], 1350 [ν(CN) C-N linkage], 1068 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.680-0.750 (m, 6H), 0.805-0.856 (m, 2H), 0.943-0.993 (m, 1H), 1.331-1.349 (m, 2H), 1.510-1.575 (m, 1H), 1.848-1.957 (m, 2H), 1.994-2.063 (m, 1H), 3.068-3.344 (m, 2H), 3.605-3.635 (m, 2H), 4.419-4.472 (m, 1H), 4.878-4.935 (m, 1H), 7.188-7.275 (m, 6H), 7.840-7.878 (m, 4H), 8.085-8.105 (d, 3H, *J* = 8 Hz), 8.132-8.191 (m, 2H), 8.270-8.290 (m, 1H), 8.715-8.734 (d, 1H, *J* = 7.6 Hz). ^{13}C NMR spectrum, δ, ppm: 22.58, 24.59, 24.59, 27.68, 36.36, 38.89, 42.02, 43.36, 48.92, 51.25, 56.36, 121.69, 123.95, 125.36, 127.68, 128.30, 132.65, 132.65, 135.50, 137.02, 137.02, 141.11, 141.11, 141.11, 142.68, 146.38, 146.36, 148.26, 151.03, 154.59, 155.39, 156.98, 168.03, 172.56, 174.69, 176.68. Mass spectrum, *m/z*: 692.28 $[M]^+$. Anal. calcd. (found) % for $C_{35}H_{38}N_4O_4F_6$: C, 60.69 (60.63); H, 5.53 (5.57); N, 8.09 (8.14); O, 9.24 (9.18).

***N*-(1-((4-Methyl-2-(2-oxo-2-((1-oxo-3-phenyl-1-((pyridin-2-ylmethyl)amino)propan-2-yl)amino)ethyl)-pentyl)amino)-1-oxopropan-2-yl)-4-(trifluoromethyl)-benzamide (13d):** Yield: 52%, m.p.: 280 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3682 [ν(NH) *sec*. amine], 3263 [ν(CH) arom. ring], 1633 [ν(CO) amide carbonyl], 1467 [ν(C=C) arom. ring], 1325 [ν(CN) C-N linkage], 1128 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.780-0.751 (m, 6H), 0.760-0.830 (m, 1H), 0.932-0.983 (m, 1H), 1.237-1.368 (m, 3H), 1.499-1.565 (m, 1H), 1.856-2.102 (m, 3H), 2.775-2.953 (m, 2H), 3.012-3.115

(m, 2H), 4.315-4.380 (m, 2H), 4.405-4.499 (m, 1H), 4.591-4.648 (m, 1H), 7.073-7.122 (m, 1H), 7.159-7.286 (m, 6H), 7.656-7.715 (dq, 1H, *J* = 7.6 Hz), 7.829-7.9159 (m, 3H), 8.078-8.115 (m, 2H), 8.186-8.229 (m, 1H), 8.472-8.484 (m, 1H), 8.598-8.657 (m, 1H), 8.714-8.755 (t, 1H, *J* = 7.6 Hz). ^{13}C NMR spectrum, δ, ppm: 20.36, 25.20, 25.20, 25.89, 34.36, 36.39, 41.32, 43.95, 44.26, 47.39, 52.68, 74.06, 112.28, 120.69, 125.68, 127.21, 127.21, 128.36, 130.58, 130.58, 133.54, 133.54, 135.59, 135.59, 137.68, 138.12, 141.69, 144.29, 148.11, 160.68, 164.29, 170.62, 174.28, 176.26. Mass spectrum, *m/z*: 639.30 $[M]^+$. Anal. calcd. (found) % for $C_{34}H_{40}N_5O_4F_3$: C, 63.84 (63.79); H, 6.30 (6.34); N, 10.95 (10.99); O, 10.00 (10.06).

***N*-(1-((4-Methyl-2-(2-oxo-2-((1-oxo-3-phenyl-1-(pyridin-2-ylamino)propan-2-yl)amino)-ethyl)pentyl)amino)-1-oxopropan-2-yl)-4-(trifluoromethyl)benzamide (13e):** Yield: 60%, m.p.: 256 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3635 [ν(NH) *sec*. amine], 3207 [ν(CH) arom. ring], 1691 [ν(CO) amide carbonyl], 1485 [ν(C=C) arom. ring], 1328 [ν(CN) C-N linkage], 1109 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.751-0.807 (m, 6H), 1.333-1.351 (m, 4H), 1.526-1.615 (m, 1H), 1.799-1.959 (m, 2H), 2.058-2.078 (m, 1H), 2.696-2.762 (m, 2H), 2.788-2.994 (m, 2H), 3.115-3.169 (m, 1H), 4.436-4.472 (m, 1H), 4.893-4.920 (m, 1H), 6.686-6.727 (m, 1H), 6.933 (s, 1H), 7.167-7.275 (m, 4H), 7.626-7.670 (m, 2H), 8.085-8.106 (m, 3H), 8.173-8.213 (m, 4H), 8.256-8.308 (m, 1H), 8.715-8.753 (m, 1H). ^{13}C NMR spectrum, δ, ppm: 21.45, 24.68, 24.68, 26.02, 35.68, 38.34, 42.15, 44.98, 46.29, 53.54, 59.85, 110.35, 115.69, 126.69, 128.14, 128.14, 129.32, 131.37, 131.37, 134.52, 134.52, 135.68, 135.68, 138.92, 139.65, 142.78, 146.42, 151.68, 159.95, 168.17, 171.52, 173.32, 175.84. Mass spectrum, *m/z*: 625.29 $[M]^+$. Anal. calcd. (found) % for $C_{33}H_{38}N_5O_4F_3$: C, 63.35 (63.30); H, 6.12 (6.17); N, 11.19 (11.24); O, 10.23 (10.19).

***N*-(1-((2-(2-((1-((4-Fluorobenzyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-4-methylpentyl)amino)-1-oxopropan-2-yl)-4-(trifluoromethyl)benzamide (13f):** Yield: 58%, m.p.: 262 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3622 [ν(NH) *sec*. amine], 2980 [ν(CH) arom. ring], 1710 [ν(CO) amide carbonyl], 1483 [ν(C=C) arom. ring], 1382 [ν(CN) C-N linkage], 1066 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.707-0.823 (m, 7H), 0.923-1.049 (m, 1H), 1.351-1.366 (m, 3H), 1.513-1.579 (m, 1H), 1.800-1.985 (m, 2H), 2.006-2.086 (m, 1H), 2.750-2.905 (m, 2H), 2.958-3.087 (m, 2H), 4.236-4.261 (m, 2H), 4.437-4.490 (m, 1H), 4.532-4.590 (m, 1H), 7.063-7.119 (m, 2H), 7.162-7.204 (m, 3H), 7.219-7.252 (m, 4H), 7.833-7.903 (m, 3H, *J* =8 Hz), 8.077-8.110 (m, 2H), 8.141-8.181 (t, 1H, *J* = 8 Hz), 8.496-8.560 (m, 1H), 8.705-8.749 (m, 1H). ^{13}C NMR spectrum, δ, ppm: 22.52, 23.17, 23.17, 29.52, 34.36, 39.68, 41.85, 43.59, 44.29, 47.02, 49.94, 53.15, 111.35, 111.35, 123.68, 127.21, 127.21, 129.65, 131.71, 131.71, 133.65, 133.65, 135.93, 135.93, 136.32, 136.32, 138.67, 140.57, 142.69, 145.92, 164.46, 169.80, 170.12, 173.57, 176.53. Mass spectrum, *m/z*: 656.30 $[M]^+$. Anal. calcd. (found) % for $C_{35}H_{40}N_4O_4F_4$: C, 64.01 (64.07); H, 6.14 (6.09); N, 8.53 (8.48); O, 9.75 (9.79).

3-((2-(2-(4-Methoxyphenyl)acetamido)propanamido)-methyl)-5-methyl-N-(1-oxo-3-phenyl-1-((pyridin-2-yl-methyl)amino)propan-2-yl)hexanamide (13g): Yield: 56%, m.p.: 274 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3682 [ν(NH) *sec*. amine], 2927 [ν(CH) arom. ring], 1631 [ν(CO) amide carbonyl], 1546 [ν(C=C) arom. ring], 1365 [ν(CN) C-N linkage]. ^{1}H NMR spectrum, δ, ppm: 0.717-0.769 (m, 7H), 0.788-0.822 (m, 1H), 0.914-0.991 (m, 2H), 1.173-1.242 (m, 3H), 1.477-1.556 (m, 1H), 1.826-1.930 (m, 2H), 1.979-2.027 (m, 1H), 2.756-2.891 (m, 3H), 3.019-3.092 (m, 2H), 3.383-3.419 (m, 2H), 4.212-4.283 (m, 1H), 4.321-4.428 (m, 2H), 4.589-4.646 (m, 1H), 6.826-6.848 (d, 2H, *J* = 8.8 Hz), 7.073-7.128 (m, 1H), 7.157-7.202 (m, 3H), 7.210-7.289 (m, 5H), 7.677-7.758 (m, 1H), 7.799-7.828 (t, 1H, *J* = 6 Hz), 8.104-8.142 (m, 1H), 8.171-8.204 (m, 1H), 8.474-8.490 (d, 1H, *J* = 8 Hz), 8.621-8.661 (m, 1H). ^{13}C NMR spectrum, δ, ppm: 20.52, 24.61, 24.61, 26.57, 30.34, 36.19, 39.34, 42.62, 44.22, 46.95, 48.24, 53.02, 54.15, 64.32, 110.35, 114.65, 121.68, 124.59, 128.36, 130.68, 131.09, 131.09, 133.42, 133.42, 136.86, 136.86, 139.59, 144.20, 147.97, 158.06, 162.38, 170.95, 174.65, 174.89, 176.77. Mass spectrum, *m/z*: 615.34 $[M]^+$. . Anal. calcd. (found) % for $C_{35}H_{45}N_5O_5$: C, 68.27 (68.31); H, 7.37 (7.43); N, 11.37 (11.32); O, 12.99 (12.86).

***N*-(1-((4-Fluorobenzyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-((2-(2-(4-methoxyphenyl)acetamido)propan-amido)methyl)-5-methylhexanamide (13h):** Yield: 51%, m.p.: 290 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3662 [ν(NH) *sec*. amine], 3278 [ν(CH) arom. ring], 1678 [ν(CO) amide carbonyl], 1552 [ν(C=C) arom. ring], 1384 [ν(CN) C-N linkage], 1035 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.732-0.793 (m, 6H), 0.800-0.816 (m, 1H), 0.908-1.002 (m, 1H), 1.174-1.207 (m, 3H), 1.476-1.554 (m, 1H), 1.917-1.803 (m, 2H), 1.962-2.037 (m, 1H), 2.756-2.814 (m, 2H), 2.967-3.088 (m, 2H), 3.379-3.419 (m, 2H), 3.713 (s, 3H), 4.199-4.313 (m, 3H), 4.533-4.591 (m, 1H), 6.825-6.846 (d, 2H, *J* = 8.4 Hz), 7.069-7.130 (m, 2H), 7.157-7.211 (m, 5H), 7.228-7.256 (t, 4H, *J* = 6.4 Hz), 7.750-7.256 (m, 1H), 8.088-8.161 (m, 2H), 8.504-8.561 (m, 1H). ^{13}C NMR spectrum, δ, ppm: 21.12, 24.52, 24.52, 27.51, 33.82, 36.68, 42.35, 43.52, 45.57, 48.60, 49.03, 54.68, 57.80, 68.40, 114.70, 114.70, 117.45, 117.45, 125.35, 129.95, 132.05, 132.05, 136.53, 136.53, 139.17, 139.17, 142.31, 142.31, 145.36, 148.97, 160.24, 164.53, 171.68, 174.82, 175.34, 176.82. Mass spectrum, *m/z*: 632.34 $[M]^+$. Anal. calcd. (found) % for $C_{36}H_{45}N_4O_5F$: C, 68.33 (68.30); H, 7.17 (7.22); N, 8.85 (8.89); O, 12.64 (12.59).

***N*-(1-((4-Methoxybenzyl)amino)-1-oxo-3-phenyl-propan-2-yl)-3-((2-(2-(4-methoxyphenyl)acetamido)propanamido)methyl)-5-methylhexanamide (13i):** Yield: 62%, m.p.: 288 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3635 [ν(NH) *sec*. amine], 3280 [ν(CH) arom. ring], 1720 [ν(CO) amide carbonyl], 1510 [ν(C=C) arom. ring], 1342 [ν(CN) C-N linkage]. ^{1}H NMR spectrum, δ, ppm: 0.718-0.813 (m, 7H), 0.908-01.004 (m, 1H), 1.181-1.212 (m, 3H), 1.476-1.558 (m, 1H), 1.808-1.916 (m, 2H), 1.962-2.037 (m, 1H), 2.750-2.853 (m, 2H), 2.967-3.091 (m, 2H), 3.389-3.425 (m, 2H), 3.714 (s, 3H), 3.723 (s, 3H), 4.163-4.271 (m, 3H), 4.538-4.596 (m, 1H), 6.828-6.869 (m, 4H), 7.089-7.129 (m, 2H), 7.168-7.205 (m, 3H), 7.216-7.257 (m, 4H), 7.749-7.821 (t, 1H, *J* = 6 Hz), 8.090-8.140 (m, 2H), 8.437-8.480 (m, 1H). ^{13}C NMR spectrum, δ, ppm: 22.65, 25.57, 25.57, 27.36, 35.68, 39.32, 41.96, 42.96, 44.27, 45.38, 48.15, 55.14, 57.65, 58.32, 58.32, 109.54, 109.54,

114.51, 114.51, 125.47, 130.96, 131.28, 131.28, 133.70, 133.70, 135.72, 135.72, 137.97, 137.97, 139.36, 141.29, 156.12, 162.95, 169.25, 170.15, 172.72, 175.13. Mass spectrum, *m/z*: 644.36 $[M]^+$. Anal. calcd. (found) % for $C_{37}H_{48}N_4O_6$: C, 68.92 (68.96); H, 7.50 (7.47); N, 8.69 (8.64); O, 14.89 (14.93).

***N*-(1-((4-Fluorobenzyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-((2-(2-(4-fluorophenyl)acetamido)propanamido)-methyl)-5-methylhexanamide (13j):** Yield: 59%, m.p.: 278 ºC, white solid. IR spectrum (ATR, ν_{max}, cm^{-1}): 3639 [ν(NH) *sec*. amine], 3280 [ν(CH) arom. ring], 1656 [ν(CO) amide carbonyl], 1504 [ν(C=C) arom. ring], 1357 [ν(CN) C-N linkage], 1095 [ν(CF)]. ^{1}H NMR spectrum, δ, ppm: 0.713-0.814 (m, 7H), 0.887-1.018 (m, 2H), 0.120-1.241 (t, 3H, *J* = 6.4 Hz), 1.491-1.569 (m, 1H), 1.804-2.041 (m, 3H), 2.757-2.909 (m, 3H), 2.967-3.086 (m, 2H), 4.201-4.314 (m, 3H), 4.538-4.609 (m, 1H), 7.070-7.148 (m, 4H), 7.164-7.210 (m, 3H), 7.210-7.305 (m, 6H), 7.751-7.834 (dt, 1H, *J* = 6 Hz), 8.079-8.241 (m, 2H), 8.508-8.564 (m, 1H). ^{13}C NMR spectrum, δ, ppm: 21.52, 27.35, 27.35, 28.25, 34.87, 36.25, 41.44, 43.61, 44.69, 46.02, 49.29, 55.31, 65.49, 113.65, 113.65, 117.73, 117.73, 129.28, 133.21, 133.21, 135.12, 135.12, 136.71, 136.71, 139.43, 139.43, 141.83, 146.80, 154.30, 164.92, 163.29, 170.62, 171.12, 174.14, 176.17. Mass spectrum, *m/z*: 620.32 $[M]^+$. Anal. calcd. (found) % for $C_{35}H_{42}N_4O_4F_2$: C, 67.72 (67.75); H, 6.82 (6.86); N, 9.03 (9.00); O, 10.31 (10.27).

Cell lines and cell culture: Human breast adenocarcinoma (MCF-7) cell line was obtained from National Center for Cell Science (NCCS), Pune, India. MCF-7 cells were cultured in DMEM medium containing L-glutamine (2 mmol/L), supplemented with 10% FBS and 1% PSN, an antibiotic mixture (Life technologies, USA). The cells were cultured in a humidified condition of 5% CO_2 at 37 ºC. The exponentially growing cells were used in entire study.

Treatments of compounds: Compounds were freshly prepared in cell culture grade DMSO at the stock concentration of 100 mM. Exponentially growing MCF-7 cells were treated with different compounds (100 µM) for 24 h. Cells treated with DMSO (0.1%) were considered as vehicle control.

Cell proliferation assay: The cell proliferation was examined by MTT assay as describe earlier [19]. Briefly, 2×10^4 MCF-7 cells were treated with series of synthesized compounds for 24 h. Thereafter, the cells were washed with DPBS and incubated with MTT (0.5 mg/mL) for 4 h in dark at 37 ºC. After incubation period the MTT was removed and DMSO was added to each well. The absorbance was recorded at 570 nm with the reference wavelength of 650 nm by using Multimode microplate reader (SpectraMax M2e, Molecular Devices, USA). The results were represented as percentage of cell proliferation.

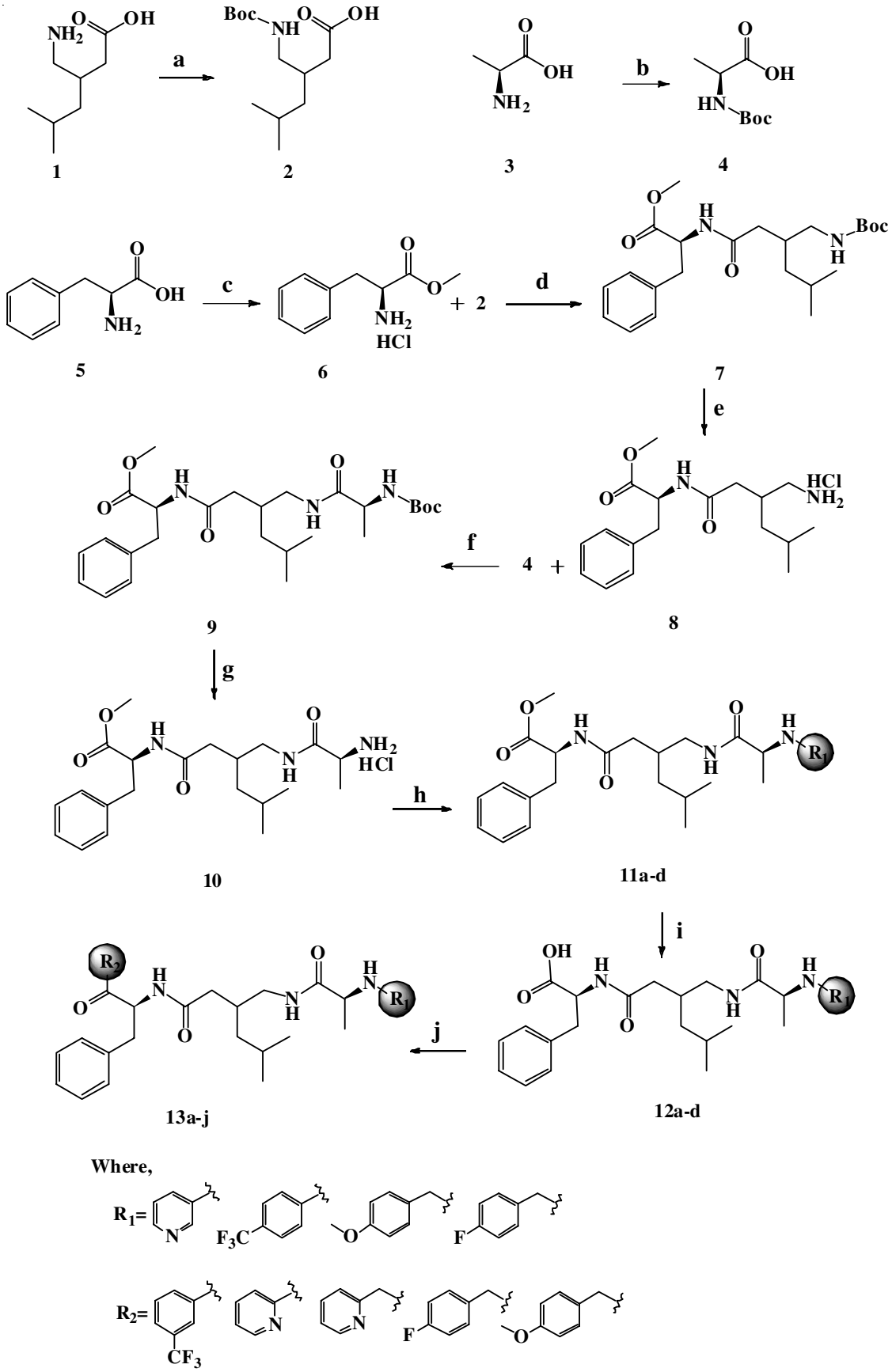
Statistical analysis: The data represented were analyzed by student t test using Sigma Stat 2.0 statistical analysis software. The normality of data was tested by Shapiro-Wilk test prior to student t test. The p values $***p \leq 0.001$ were considered as statistically significant.

RESULTS AND DISCUSSION

Synthetic strategies adopted to obtain the target compounds are depicted in **Scheme-I**. The series of title compounds **(13a-j)** were synthesized in ten steps. Compounds **2** and **4** formed by pregabalin and L-alanine, respectively by reaction with NaOH and Boc (di-*tert*-butyldicarbonate) at 0-5 ºC followed by 12 h room temperature stirring. (Herein, we used the overnight stirring for 12 h at room temperature. On the other hand, **Int. 6** was synthesized in solvent methanol using L-phenyl alanine (**5**) and dropwise addition of $SOCl_2$ in the temperature range between 0 ºC room temperature and the yield reached more than 60%. Compound **7** was prepared by a reaction of **Int. 2** and **6** in the presence of $C_2H_4Cl_2$ and DMAP in the temperature ranges between 0 ºC to room temperature using DMF as a solvent. Intermediate **7** was dissolved in DCM followed by acidified with cold HCl and then stirred for 3-4 h at room temperature to obtain compound **8**. The diverse targeted motif, *i.e.* tripeptide (**9**), was formed by coupling of **4** and **8** (HCl salt) in the presence of $C_2H_4Cl_2$ and DMAP at room temperature, stirred overnight. This intermediate (**Int. 9**) reacts with HCl to form tripeptide HCl salt (**Int.10**). **Int. 11a-d** was synthesized from N-terminal alkylation of compound **10** in the presence of $C_2H_4Cl_2$ and DMAP below 5 ºC. Basic hydrolysis of compounds **11a-d** was carried out in THF, using LiOH as base and stirred at room temperature overnight to form compounds **12a-d**. In the final step, compounds **(13a-j)** were generated from the intermediate **(12a-d)** by the reaction of DMAP/$C_2H_4Cl_2$ at room temperature.

The structure of synthesized peptides **13a-j** was confirmed based on the spectral data. The IR spectrum of compound **13a-j** showed a strong absorption band at ~3682 cm^{-1} due to N-H stretching, secondary amine. The absorption band appeared at ~3263 cm^{-1} due to stretching vibrations of aromatic hydrogen. The intense absorption peaks at ~1633 cm^{-1} were obtained due to >C=O stretching in amide carbonyl. Moreover, absorption bands at ~1512, ~1350 cm^{-1} corresponding to C=C, C-N stretching, respectively. Compound **13a-j** showed a characteristic peak at ~1068 cm^{-1} assignable to the C-F bond. In ^{1}H NMR spectra of the compounds **13a-j,** the protons of the terminal methyl group obtained at δ = ~0.75 ppm. The presence of -CO-C<u>H</u>-NH- linkage showed a multiplet peak at ~4.41 ppm. Protons of phenyl ring gave a multiplet at δ = ~7.06 to ~8.56 ppm. The absorption peak at δ = ~8.71 executed a proton of secondary amine in the structure. The remaining substituent protons were in good agreement with theoretical values. ^{13}C NMR spectra assisted to identify the formation of the final adducts. The characteristic value δ = ~20 to ~25 ppm showed the presence of methyl group present in the title compounds. The signal obtained at δ = ~46 ppm confirmed the presence of methylene carbon between the isobutyl group and amide linkage. The aromatic ring carbons were in decent covenants with the theoretical values (δ = ~125 ppm). The characteristic value at δ = ~176 ppm showed carbonyl carbon (>C=O) in compounds **13a-j**. The mass spectrum revealed a molecular ion peak in compound **13a-j** at *m/z* = ~558 to ~692 in mass spectra; molecular ion peaks were in good agreement with the proposed molecular weight and elemental analysis.

Anticancer screening against MCF-7 cancer cell line: Ten synthesized pregabalin based peptides derivatives were evaluated for their *in vitro* anticancer activity on the human



Scheme-I: Synthesis pathway of pregabalin-amino acids hybrid peptides (**13a-j**); **Reaction condition:** (a) Boc anhydride, NaOH/H_2O, 0 °C - RT, 12 h; (b) Boc anhydride, NaOH/H_2O, 0 °C - RT, 12 h; (c) MeOH, $SOCl_2$, 0 °C - RT, 12 h; (d) EDC·HCl, DMAP, DMF, 0 °C - RT, 12 h; (e) DCM, HCl, 0 °C - RT, 3-4 h; (f) DMF, EDC·HCl, DMAP, 0 °C - RT, 12 h; (g) DCM, HCl, 0 °C - RT, 3-4 h; (h) DMF, EDC·HCl, DMAP, 0 °C - RT, 12 h; (i) LiOH, THF, H_2O, RT, Overnight; (j) EDC·HCl, DMAP, DMF, 0 °C - RT, 12 h

cancer cell line, particularly MCF-7 (breast cancer) obtained from the Cell repository, National Center for Cell Science, Pune, Maharashtra, India [19,20].

Herein, we observed that only one molecule **13a** was more active than their corresponding peptides. The biological effect is dependent on the substituent present on R_1 and R_2 (C & N terminal sides). However, a clear structure-activity relationship between the size/substitutions of the moiety and the anti-proliferative effect of the MCF-7 human breast cancer cell line is not observed. The most active compound **13a**, which presents a 3-pyridyl and 3-trifluoro phenyl group as a substituent at two terminal of peptide core, shows significant results with %cell proliferation, 16.65% and net cell death 83.35%. None of the other chosen substituents was shown remarkable development towards efficacy in the MCF-7 cell line. In conclusion, literature-based active functionalities such as pyridine, CF_3-aryl, F-aryl, methoxybenzene [21-25] have been selected for enhancing the potency of peptides but surprisingly only one compound (**13a**) shown excellent cell proliferation against MCF-7 cell line. At this point, it has now necessary for additional research to better guide further modification and application development of specific ACPs. Our recent finding and future perspectives show that palmitoylation of ACPs can effectively reduce their cytotoxicity and their antitumour activity could be significantly improve [16,26] (Table-1 and Fig. 1).

TABLE-1
RESULT OF ANTICANCER ACTIVITY OF SYNTHESIZED COMPOUNDS **(13a-j)** BY MTT ASSAY METHOD

Entry	% Cell proliferation	% Cell death (antiproliferation)
13a	16.65	83.35
13b	63.62	36.38
13c	66.59	33.41
13d	53.80	46.20
13e	62.64	37.36
13f	62.87	37.13
13g	44.63	55.37
13h	56.21	43.79
13i	54.13	45.87
13j	65.82	34.18

Conclusion

In this study, novel pregabalin based derivatives incorporated to different chiral amino acids (**13a-j**) were synthesized using solution phase method in peptide synthesis through ten step procedures. All novel compounds were evaluated for their cytotoxic activity upon MCF-7 cell line using MTT assay. Compound **13a** exhibited the most significant suppression of the proliferation of the cancer cells and may be considered as a suitable lead for further development of anticancer drugs in future. The spatial orientation and efficacy based on substituents is the topics for further research in the area of *in vivo* studies on the compounds for the development of new pharmaceutical drugs.

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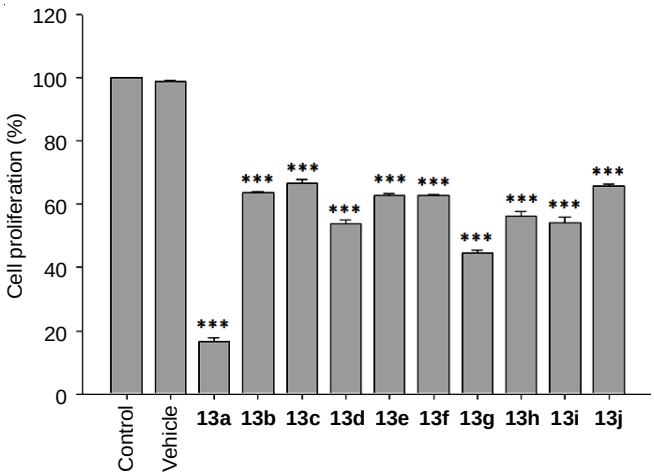

Fig. 1. Evaluation of cell proliferation by MTT assay for synthesized compounds **(13a-j)** at 100 μM concentrations. The bar graphs represent the percentage of cell proliferation. Vehicle control contained 0.1% DMSO and control represents untreated cells. Error bars represent ± SEM of three independent experiments. Significance indicated as ***$p \leq 0.001$ between untreated cells and treated cells

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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