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# Anionic diketopiperazine induces osteogenic differentiation and supports osteogenesis in 3D cryogel microenvironment

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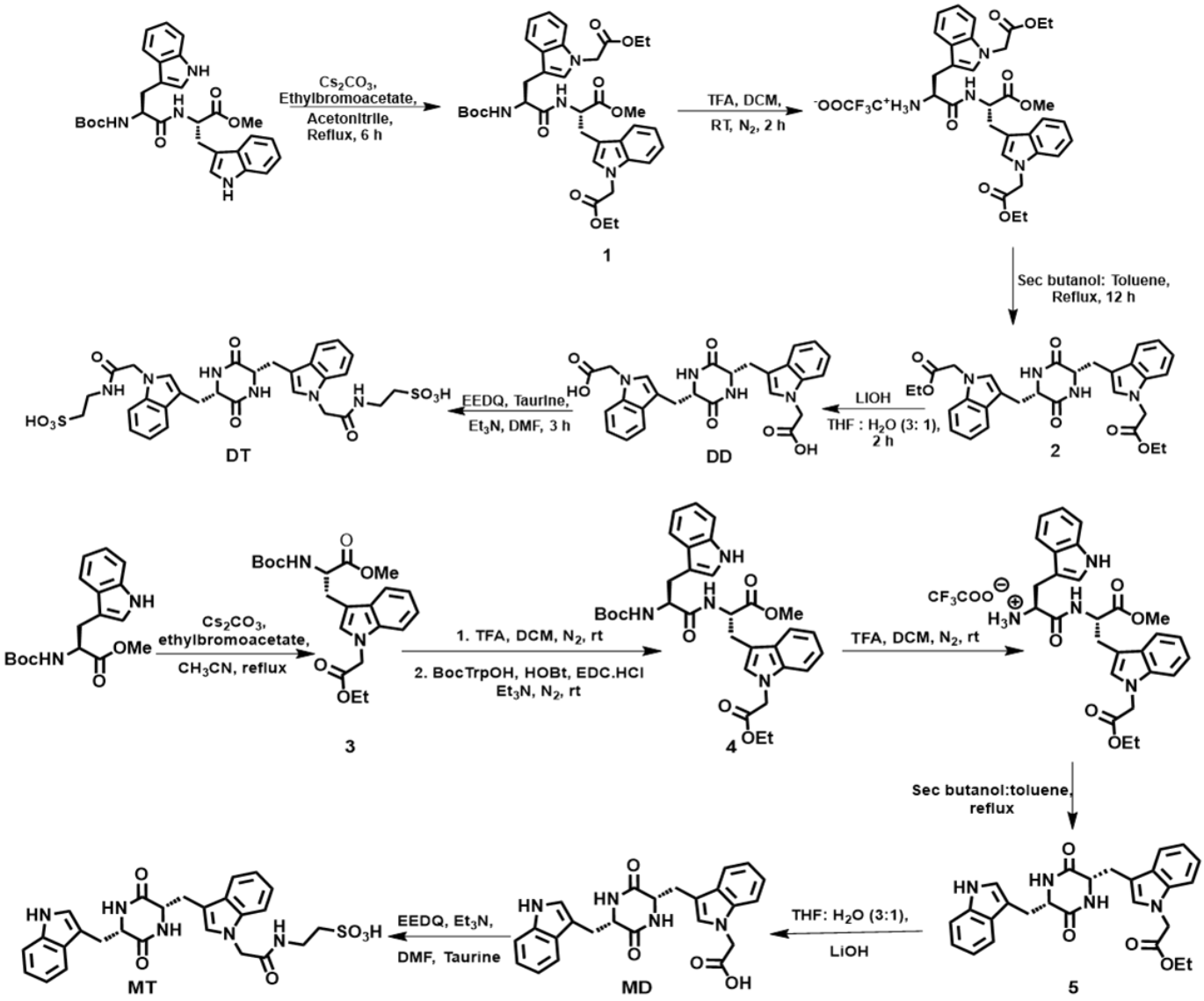
## 1. Scheme




**Scheme 1**: Synthetic scheme for diketopiperazines, **DT** and **MT**.

## 2. Materials and Methods

2.1 Materials: Acetonitrile ($CH_3CN$), dichloromethane (DCM), methanol (MeOH) were distilled following standard procedures. Boc anhydride, taurine, ethylbromoacetate and thionyl chloride were obtained from Spectrochem (Mumbai, India). L-tryptophan, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 1-hydroxybenzotriazole, trifluoroacetic acid, N-methylmorpholine were purchased from Avra Synthesis Pvt. Ltd. (Hyderabad, India). Sodium bicarbonate, HPLC-grade water, DMSO-$d_6$ and sodium chloride were purchased from Merck. Hydrochloric acid from Fischer scientific. N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was purchased from TCI Chemicals (India) Pvt. Ltd. Amberlite Resin, triethylamine were obtained from S. D. Fine Chem. Limited (Mumbai, India).

2.2 NMR and HRMS-ESI

$^1H$ and $^{13}C$ NMR spectra were recorded on JEOL-JNM ECS 400/500 model operating at 400/500 MHz and 100/125 MHz, respectively. HRMS spectra were recorded at IIT Kanpur, India, on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 kV.

2.3 High-Performance Liquid Chromatography (HPLC). HPLC analysis was performed on HPLC system (Agilent Technologies 1260 infinity) equipped with a quaternary pump (G1311B), auto liquid sampler (G1329B), diode array detector (G1315D) and analytical scale-fraction collector (G1364C). Instrument control, data acquisition and data analysis were performed using ChemStation software (Agilent Technologies, Wokingham, UK). A ZORBAX Eclipse plus from C18 (250 x 4.6 mm) column with 5 µm particle size at room temperature from Agilent technologies was used for compounds DD, MT, and DT. The mobile phase consisted of acetonitrile/water with 0.1% TFA, and the flow rate was 1.0 mL/min. Injection volume was 10 µL and the column effluent was monitored at 280 nm.

2.4 Synthesis and Characterization of Compounds.

The designed diketopiperazines were synthesized using solution phase synthesis protocol.

The Boc-di-L-Tryptophan and Boc-L-Tryptophan methyl ester were synthesized using literature method.[1]

Synthesis of compounds:

*Synthesis of methyl Nα-(Nα-(tert-butoxycarbonyl)-1-(2-ethoxy-2-oxoethyl)-L-tryptophyl)-1-(2-ethoxy-2-oxoethyl)-L-tryptophan 1*: The Boc-di-L-Tryptophan methyl ester (2 g, 3.96 mmol) was dissolved in acetonitrile (30 mL). $Cs_2CO_3$ (7.75 g, 23.78 mmol) was then added to the solution, followed by addition of ethyl bromoacetate (2.63 mL, 23.78 mmol). The reaction mixture was refluxed for 12 h at 100 °C. The crude product was re-dissolved in dichloromethane and washed with water. The product was purified using column chromatography with dichloromethane and methanol as solvent system (99:1). (Yield: 82.01 %) $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.36 (d, J = 7.5 Hz, 1H), 7.63 (d, J = 7.8 Hz, 1H), 7.53 (d, J = 7.8 Hz, 1H), 7.35 – 7.30 (m, 2H), 7.20 – 6.98 (m, 6H), 6.79 (d, J = 8.3 Hz, 1H), 5.04 (s, 2H), 5.02 (s, 2H), 4.58 (q, J = 7.4 Hz, 1H), 4.28 – 4.23 (m, 1H), 4.15 – 4.08 (m, 4H), 3.57 (s, 3H), 3.18 (dd, J = 14.8, 6.2 Hz, 1H), 3.09 (dd, J = 14.8, 7.8 Hz, 1H), 3.03 (dd, J = 14.7, 3.9 Hz, 1H), 2.89 (dd, J = 14.6, 9.7 Hz, 1H), 1.31 – 1.18 (m, 15H). $^{13}C$ NMR (125 MHz, DMSO-$d_6$): $\delta$ 172.16, 172.03, 168.92, 155.44, 136.50, 127.91, 127.81,

127.58, 121.39, 121.25, 118.96, 118.82, 118.67, 118.31, 109.77, 109.59, 78.08, 60.82, 54.92, 52.79, 51.83, 46.91, 28.09, 14.05. HRMS- ESI (m/z) calculated for $C_{36}H_{44}N_4O_9$, $[M+NH_4]^+$ = 694.3447, found = 694.3452.

*Synthesis of diethyl 2,2'-((((2S,5S)-3,6-dioxopiperazine-2,5-diyl)bis(methylene))bis(1H-indole-3,1-diyl))diacetate 2:* The compound 1 (1 g, 1.47 mmol) was dissolved in 30% TFA in dichloromethane (20 mL) and stirred at room temperature under $N_2$ atmosphere. Solvent was evaporated under high vacuum and washed with diethylether. The crude solid was dissolved in *sec*-butanol (240 mL) and toluene (80 mL). The solution was refluxed at 140 °C. After concentrating the solution to 5-10 mL and cooling to 0 °C, the product was filtered off and dried in high vacuum. The product was purified by column chromatography with 4% methanol-dichloromethane as solvent system. (Yield: 74%). $^1$H-NMR (500 MHz, DMSO-$d_6$): $\delta$ 7.66 (d, J = 2.0 Hz, 2H), 7.34 (d, J = 7.8 Hz, 2H), 7.25 (d, J = 8.2 Hz, 2H), 7.06 (t, J = 7.3 Hz, 2H), 6.98 (t, J = 7.4 Hz, 2H), 6.66 (s, 2H), 4.94 (s, 4H), 4.09 – 4.04 (m, 4H), 3.87 (s, 2H), 2.72 (dd, J = 14.3, 4.1 Hz, 2H), 2.29 (dd, J = 14.5, 6.5 Hz, 2H), 1.14 (t, J = 7.1 Hz, 6H). $^{13}$C-NMR (500 MHz, DMSO-$d_6$): $\delta$ (ppm) $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 168.87, 166.61, 136.55, 128.69, 127.91, 121.24, 118.93, 109.66, 109.19, 60.84, 55.16, 46.91, 40.02, 39.85, 39.69, 39.52, 39.35, 39.19, 39.02, 29.85, 14.06. HRMS – ESI (m/z) calculated for $C_{30}H_{32}N_4O_6$, $[M+H]^+$ = 545.2395, found = 545.2397.

*Synthesis of 2,2'-((((2S,5S)-3,6-dioxopiperazine-2,5-diyl)bis(methylene))bis(1H-indole-3,1-diyl))diacetic acid DD:* The 2,2'-((((2S,5S)-3,6-dioxopiperazine-2,5-diyl)bis(methylene))bis(1H-indole-3,1-diyl))diacetate (400 mg, 0.73 mmol) was dissolved in THF (6 mL). Then, a solution of LiOH.$H_2O$ (61.2 mg, 1.46 mmol) in distilled water (2 mL) was added and stirred at room temperature for 2 h. The solution obtained was passed over cation exchange resin. The filtrate was evaporated under reduced pressure to obtain pure product. The product was purified by column chromatography with dichloromethane and methanol as solvent system (92:8). (Yield: 75.25 %). $^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.65 (d, J = 2.2 Hz, 2H), 7.36 (d, J = 7.9 Hz, 2H), 7.28 (d, J = 8.2 Hz, 2H), 7.09 (t, J = 7.6 Hz, 2H), 7.01 (t, J = 7.4 Hz, 2H), 6.73 (s, 2H), 4.87 (s, 4H), 3.93 – 3.90 (m, 2H), 2.77 (dd, J = 14.5, 4.2 Hz, 2H), 2.34 (dd, J = 14.5, 6.7 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 170.84, 167.17, 137.09, 129.24, 128.43, 121.68, 119.45, 119.32, 110.14, 109.51, 55.74, 47.48, 30.33. HRMS-ESI- (m/z) calculated for $C_{26}H_{24}N_4O_6$, $[M+H]^+$ = 489.1769, found = 489.1777.

*Synthesis of 2,2'-((2,2'-((((2S,5S)-3,6-dioxopiperazine-2,5-diyl)bis(methylene)) bis(1H-indole-3,1-diyl))bis(acetyl))bis(azanediyl))bis(ethane-1-sulfonic acid) DT*: Taurine (92.3 mg, 0.73 mmol) and triethylamine (75 µL, 0.53 mmol) were added to the stirring solution of DD (200 mg, 0.40 mmol) and EEDQ (141 mg, 0.57 mmol) in 10 mL of DMF. The reaction mixture was heated at 90 °C for 2 h and then cooled to room temperature with stirring for half an hour. The solution was poured slowly to 40 mL diethyl ether in an ice bath. The precipitates formed were dissolved in methanol and then passed over activated cation exchange resin. The product was purified by column chromatography using dichloromethane and methanol as solvent system (90:10). (Yield: 24.33 %).$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.84 (t, J = 5.5 Hz, 2H), 7.80 (d, J = 1.7 Hz, 2H), 7.34 (d, J = 7.9 Hz, 2H), 7.28 (d, J = 8.3 Hz, 2H), 7.10 (t, J = 7.4 Hz, 2H), 7.00 (t, J = 7.4 Hz, 2H), 6.73 (s, 2H), 4.80 – 4.60 (m, 4H), 3.94 – 3.85 (m, 2H), 3.32 –

3.30 (m, 4H), 2.80 (dd, J = 14.2, 3.6 Hz, 2H), 2.63 – 2.51 (m, 4H ), 2.26 (dd, J = 14.2, 7.2 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 167.32, 166.61, 136.43, 128.67, 127.98, 121.20, 118.86, 109.62, 109.02, 55.41, 50.35, 48.75, 35.62, 34.37, 29.92. HRMS-ESI- (m/z) for $C_{30}H_{34}N_6O_{10}S_2$, Calculated $[M-H]^-$ =701.1705, found = 701.1664; Calculated $[M+NH_4]^+$ = 720.2116, found = 720.2112.

*Synthesis of methyl Nα-((tert-butoxycarbonyl)-L-tryptophyl)-1-(2-ethoxy-2-oxoethyl)-L-tryptophan 4:* The Boc-L-Tryptophan methyl ester (3.15 g, 9.89 mmol) was dissolved in acetonitrile (100 mL). $Cs_2CO_3$ (6.45 g, 19.79 mmol) was then added to the solution, followed by addition of ethyl bromoacetate (2.19 mL, 19.79 mmol). The reaction mixture was refluxed for 12 h at 100 °C. The crude product was re-dissolved in dichloromethane and washed with water. The completion of reaction was monitored by TLC and product was characterized by NMR of crude sample (compound 3). The crude product 3 was dissolved 30% TFA in dichloromethane and stirred for 2 h under $N_2$ atmosphere. To a clear solution of N-(Boc)-L-tryptophan (2 g, 6.57 mmol) in dichloromethane (30 mL), 1-hydroxybenzotriazole (1.15 g, 8.54 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.64 g, 8.54 mmol) was added at 0 °C under nitrogen atmosphere. After half an hour, N-methylmorpholine (0.86 mL, 7.89 mmol) was added followed by addition of TFA salt of methyl Nα-(tert-butoxycarbonyl)-1-(2-ethoxy-2-oxoethyl)-L-tryptophan 3 (3.1 g, 7.23 mmol) and the stirring was continued for 12 h at room temperature. After completion of the reaction, the mixture was washed twice with 10% aqueous sodium bicarbonate solution and then with 1 N aqueous hydrochloric acid solution. The combined organic layers were washed with brine solution and dried over anhydrous sodium sulphate. The organic phase was subsequently evaporated under reduced pressure. The crude product was purified by silica gel column chromatography using dichloromethane and methanol as solvent system (99:1) to give target compound as white powder (Yield: 90.17%).$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.78 (s, 1H), 8.35 (d, J = 7.4 Hz, 1H), 7.67 – 7.47 (m, 2H), 7.33 (t, J = 7.3 Hz, 2H), 7.15 – 6.95 (m, 6H), 6.73 – 6.67 (m, 1H), 5.01 (s, 1H), 4.99 (s, 1H), 4.61– 4.49 (m, 1H), 4.30 – 4.20 (m, 1H), 4.13 – 4.05 (m, 2H), 3.57 (d, J = 2.9 Hz, 3H), 3.22 – 2.75 (m, 4H), 1.33 – 1.11 (m, 12H). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 172.18, 172.13, 168.96, 155.23, 136.48, 136.04, 127.92, 127.60, 127.37, 123.75, 121.42, 120.82, 118.99, 118.53, 118.31, 118.14, 111.26, 110.10, 109.80, 109.60, 78.06, 67.38, 60.84, 60.27, 54.98, 52.81, 51.86, 46.85, 28.13, 27.65, 26.84, 14.05. HRMS-ESI (m/z) calculated for $C_{32}H_{38}N_4O_7$ $[M+H]^+$ = 591.2813, found = 591.2811.

*Synthesis of ethyl 2-(3-(((2S,5S)-5-((1H-indol-3-yl) methyl)-3,6-dioxopiperazin-2-yl) methyl)-1H-indol-1-yl) acetate 5:* The compound 4 (2 g, 3.38 mmol) was dissolved in 30% TFA in dichloromethane (20 mL) and stirred for 2 h under $N_2$ atmosphere. Solvent was evaporated under high vacuum and washed with diethyl ether. The crude solid was dissolved in *sec*-butyl alcohol (240 mL) and toluene (80 mL). The solution was refluxed at 140 °C. After conc. the solution to 5-10 mL followed by cooling to 0 °C, the product was filtered off and dried in high vacuum. The crude product was purified by silica gel column chromatography using dichloromethane and methanol as solvent system (97:3) to give target compound (Yield: 81%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.79 (s, 1H), 7.73 (d, J = 2.5 Hz, 1H), 7.56 (d, J = 2.4 Hz, 1H), 7.40 – 7.21 (m, 4H), 7.07 – 6.90 (m, 4H), 6.65 (d, J = 2.2 Hz, 1H), 6.50 (s, 1H), 4.91 (s, 2H), 4.06 (q, J = 7.1 Hz, 2H), 3.90 – 3.80 (m, 2H), 2.75 – 2.62 (m, 2H), 2.37 (dd, J = 14.4, 6.3 Hz, 1H), 2.08 (dd, J = 14.4 Hz, 6.9 Hz, 1H), 1.14 (t, J = 7.1 Hz, 3H). $^{13}$C-NMR (125 MHz, DMSO-$d_6$) $\delta$ 168.49, 166.56, 166.49,

136.44, 135.94, 128.39, 127.69, 127.32, 124.26, 121.07, 120.58, 118.72, 118.68, 118.47, 118.16, 111.09, 109.37, 109.12, 108.74, 60.60, 55.19, 54.94, 46.86, 29.75, 29.61, 13.81. HRMS-ESI- (m/z) calculated for $C_{26}H_{26}N_4O_4$ $[M+Na]^+$ = 481.1846, found = 481.1858.

*Synthesis of 2-(3-(((2S,5S)-5-((1H-indol-3-yl)methyl)-3,6-dioxopiperazin-2-yl)methyl)-1H-indol-1-yl) acetic acid MD:* The compound 5 (412 mg, 0.89 mmol) was dissolved in THF (6 mL). Then, a solution of LiOH.$H_2O$ (97.24 mg, 2.3 mmol) in distilled water (2 mL) was added. The mixture was stirred at room temperature for 2 h. The solution obtained was passed over cation exchange resin. The filtrate was evaporated under reduced pressure to obtain pure product. The crude product was purified with column to give target compound. (Yield: 72.39 %) .$^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.83 (s, 1H), 7.77 (s, 1H), 7.55 (s, 1H), 7.41 (d, J = 7.7 Hz, 1H), 7.32 – 7.27 (m, 3H), 7.10 – 6.96 (m, 4H), 6.73 (s, 1H), 6.53 (s, 1H), 4.85 (s, 2H), 3.93 (s, 1H), 3.87 (s, 1H), 2.79 (dd, J = 14.3, 3.9 Hz, 1H), 2.69 (dd, J = 14.2, 3.8 Hz, 1H) 2.11 – 2.05 (m, 2H). $^{13}$C-NMR (125 MHz, DMSO-$d_6$): $\delta$ 170.25, 166.66, 136.56, 136.02, 128.67, 127.76, 127.46, 124.47, 121.11, 120.77, 118.74, 118.67, 118.35, 111.29, 109.58, 108.89, 108.81, 55.31, 55.09, 46.93, 29.88. HRMS-ESI (m/z) calculated for $C_{24}H_{22}N_4O_4$ $[M+H]^+$ = 431.1714, found = 431.1710

Synthesis of 2-(2-(3-(((2S,5S)-5-((1H-indol-3-yl) methyl)-3,6-dioxopiperazin-2-yl) methyl)-1H-indol-1-yl) acetamido) ethane-1-sulfonic acid MT: Taurine (52 mg, 0.418 mmol) and triethylamine (84 µL, 0.604 mmol) were added to the stirring solution of MD (200 mg, 0.47 mmol) and EEDQ (160 mg, 0.65 mmol) in 10 mL of DMF. The reaction mixture was heated at 90 °C for 2 h and then cooled to room temperature with stirring for half an hour. The solution was poured slowly to 40 mL diethyl ether in an ice bath. The precipitates formed were dissolved in methanol and then passed over activated cation exchange resin. The crude product was purified with column chromatography using dichloromethane and methanol as solvent system (90:10), (Yield: 54 %). $^1$H NMR (500 MHz, DMSO- $d_6$) δ 10.95 (s, 1H), 7.99 (s, 1H), 7.74 (t, J = 5.4 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.32 (d, J = 8.1 Hz, 1H), 7.26 (d, J = 8.3 Hz, 1H), 7.14 (d, J = 7.8 Hz, 1H), 7.11 – 7.03 (m, 2H), 7.00 – 6.88 (m, 3H), 6.37 (s, 1H), 4.73 – 4.58 (m, 2H), 4.04 – 3.98 (m, 1H), 3.79 – 3.71 (m, 1H), 3.36 – 3.29 (m, 2H), 2.90 – 2.77 (m, 2H), 2.66 – 2.51 (m, 3H), 1.62 – 1.55 (m, 1H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 167.39, 166.73, 166.63, 136.51, 136.05, 128.43, 127.76, 127.70, 124.73, 121.19, 120.92, 118.98, 118.83, 118.53, 118.49, 111.45, 109.59, 109.23, 108.68, 55.51, 55.26, 50.38, 48.77, 35.62, 30.21, 29.65. HRMS-ESI- (m/z) Calculated for $C_{26}H_{27}N_5O_6S$, $[M+H]^+$ = 538.1755, found = 538.1764.

2.5 Field emission scanning electron microscopy (FESEM)

A 10 µL aliquot of DT (1 mM) was placed on copper surface and was allowed to dry at room temperature for 24 h followed by drying under high vacuum for another 30 minutes. Field emission scanning electron microscopy (FESEM) images were acquired on FEI QUANTA 200 microscope, equipped with a tungsten filament gun, operating at working distance 6 mm and 10.0 kV.

2.6 Biological assays

The cellular evaluation was carried out using murine pre-osteoblasts (MC3T3E1) and rat bone marrow derived mesenchymal stem cells (BMSCs). MC3T3E1 cells were cultured in α-MEM with 10% v/v FBS and 1% penicillin–streptomycin antibiotic cocktail under a 5% $CO_2$ environment in a humidified incuba-

tor while as for BMSCs DMEM with 20% v/v FBS and 1% penicillin–streptomycin antibiotic cocktail was used. Cells were trypsinized using trypsin-EDTA (0.25% trypsin, 0.02% EDTA) and a viable cell count was confirmed manually using hemocytometer.

2.6.1 Cell proliferation assay

Cell proliferation was carried out using MTT assay. The cells were seeded in 24 well plate at a density of $1 \times 10^5$ cells per well and allowed to adhere for 24 hours. After 24 h, MC3T3E1 cells were treated with different concentrations of taurine, DD, and DT with DMSO as control vehicle. In positive control we used osteogenic media containing 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid. The media was changed after every three days with fresh treatment of the compounds. The cell viability and proliferation of MC3T3E1 cells at different concentration of the compounds was evaluated using MTT at a concentration of 0.5 mg/mL at 1, 3, 5, 7, 10 &15 days of the culture. For BMSCs, only one concentration of taurine, MT and DT was used (100 µM) for cell proliferation assay based on the screening results obtained with MC3T3E1 cells. Moreover, the cell viability was also evaluated by live/dead assay using Calcein AM and PI staining at day 3 and day 7.

2.6.2 Cell differentiation assay

The osteogenic differentiation of the cells was carried out by measuring the alkaline phosphatase activity (ALP) and alizarin red staining (ARS). ALP is an early marker of osteogenic differentiation whereas calcium deposition occurs during the late phase of differentiation.

2.6.3 Alkaline phosphatase activity

A well-established method which uses p-nitrophenol phosphate (p-NPP) as substrate was used for ALP assay as described elsewhere.[2] Briefly, at specific time points the cells were washed thrice with PBS followed by addition of transparent p-NPP substrate. The cells were incubated for 45-60 minutes at 37℃ until the yellow color appears. The absorbance was taken at 405 nm. For measuring ALP activity, MC3T3E1 cells were treated with different concentration of the compounds and ALP was measured at 1, 3, 5, 7, 10 & 15 days of the culture. For BMSCs, 100 µM of the compounds was used for measuring ALP at 1, 3, 5, 7, 10 & 15 days.

2.6.4 Alizarin red staining

MC3T3E1 cells were seeded at a density of $1 \times 10^5$ cells/well and were allowed to adhere for 24 h before treatment. The cells were treated with 100 µM of taurine, DMSO, DD & DT. The media was changed after every 3$^{rd}$ day including fresh treatment of the compounds. Calcium deposition was measured by alizarin staining at day 7, day 14 and day 21 post-treatment, while un-treated cells were used as control. Pre-osteoblast MC3T3E1 cells cultured in osteogenic medium were used as positive control for maturation and calcium deposition.

2.7 Synthesis and characterization of macroporous composite cryogel scaffolds

The macroporous composite cryogel was synthesized by cryogelation as described elsewhere.[3] Briefly, 1% collagen and 4% gelatin were dissolved in degassed acetic acid followed by addition of 20% nano-hydroxyapatite (nHAP). The mixture was mixed thoroughly followed by addition of 0.5% glutaraldehyde

as crosslinker. The blend was poured in 2 mL syringe molds at frozen at -15 ℃ for 12 hours. After complete polymerization, the cryogels formed were subjected to freeze-thawing cycles three times in $dH_2O$ to remove the unreacted crosslinker followed by lyophilization for drying and storage for future use. The synthesized cryogels were characterized by scanning electron microscopy (SEM) and FTIR spectroscopy to show the macroporous architecture, crosslinking and incorporation of nHAP crystals.

2.7.1 Cell-material interactions

The composite cryogel scaffolds were cut into thin discs of size 8 mm diameter × 2 mm height followed by sterilization in increasing ethanol gradient and allowed to dry. The scaffolds were then washed with 1X PBS three times with each washing for 15 min. MC3T3E1 cells were trypsinized, counted for viability and centrifuged to achieve the desired cell number. DT was dissolved in methanol and 1 mM (10 times higher than the concentration used for 2D experiments) of DT was added onto the cryogels in non-treated 24 well plate and the volume added was such that DT solution doesn't leak from the scaffolds. The scaffolds were then incubated for an hour in a humidified incubator before cell seeding. In case of the cryogel (CG) only group, the scaffolds were incubated with same volume of methanol. A total of $5 \times 10^5$ cells were seeded on each scaffold contained in 15 µL of culture medium and the culture plates were transferred to a humidified incubator for 4 hours to allow cell adhesion after which additional media of 500 µL was added. In CG+OM and CG+DT+OM, osteogenic media was used. The osteogenic media consisted of αMEM with 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid, 10% FBS and 1% antibiotic cocktail. Media was changed every third day and fresh osteogenic media was added. At each time point of day 3, 5, 10 & 15. Exhausted media was aspirated, and the scaffolds were washed with PBS thrice followed by the addition of 500 µL MTT reagent. The scaffolds were then incubated at 37 °C in a humidified incubator for 4 h to allow the formation of formazan crystals. The purple crystals were dissolved in 500 µL DMSO for 30 min and the absorbance was taken at 570 nm using BioTek spectrophotometer. Further at day 3 and day 7, the cell viability on CG and CG + DT was assessed by live/dead assay using Calcein AM and PI staining.

2.7.2 Cell adhesion

Scanning electron microscopy was done to evaluate the cell adhesion on the cryogels functionalized with DT. At day 7, the CG and CG + DT scaffolds were washed with 1X PBS and the cells were fixed using 4 % paraformaldehyde for 30 minutes at room temperature. The scaffolds were washed with PBS thrice post-fixation and dried in desiccator. For obtaining SEM micrographs the fixed samples were sputter coated with gold before analyzing in SEM instrument (JSM-6010LA, JEOL).

2.7.3 Alkaline Phosphatase activity

The cryogel scaffolds were functionalized with DT as described above followed by cell seeding at the same density of $5 \times 10^5$ cells as used for MTT. Four groups of scaffolds were used for measuring ALP activity: CG, CG + OM, CG + DT and CG + DT + OM. In osteogenic media containing groups, αMEM with 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid, 10% FBS and 1% antibiotic cocktail was used. However, to minimize the color interference, phenol-red free media was used for measurement of ALP activity. At each time point (day 3, day 5, day 10 and day 15), the media was removed, and cells were

washed with 1X PBS thrice. After washing, 500 µL of p-NPP was added to the scaffolds followed by incubation at 37° C for 60 minutes. Thereafter, the absorbance was measured in flat-bottom 96-well plates at 405 nm.

### 2.7.4 Statistical analysis

All the data analysis was carried out using GRAPHPAD PRISM and reported as Mean ± SEM.
Statistical significance ($P < 0.05$) was calculated using two-way ANOVA with a Tukey post hoc test.

## 3. Figures and graphs

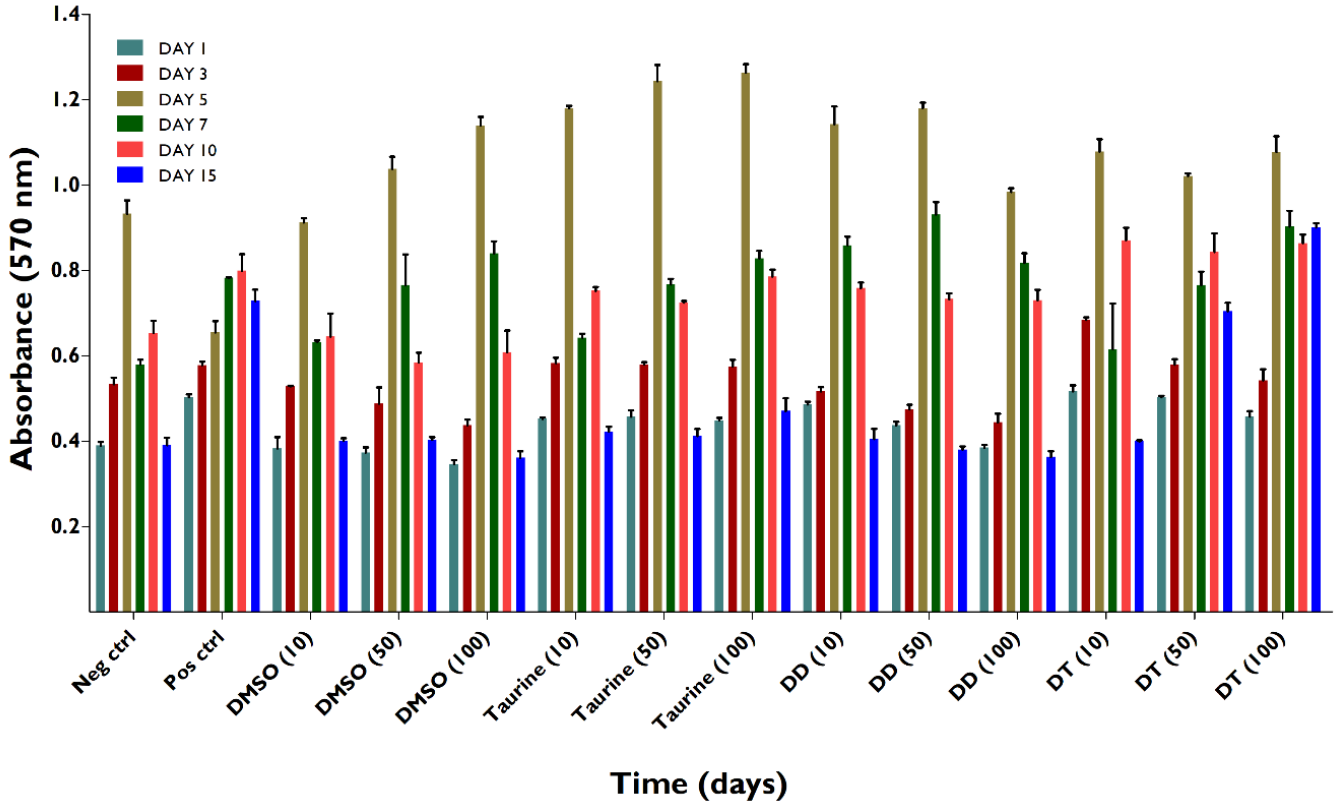



**Figure S1**: Figure shows MTT assay of MC3T3E1 into osteogenic lineage in a dose-dependent fashion with all the controls taken into consideration for 15 days (Negative control: untreated, DMSO: vehicle solvent, Positive control: osteogenic media). The value in parentheses indicates the concentration of compounds tested in µM.

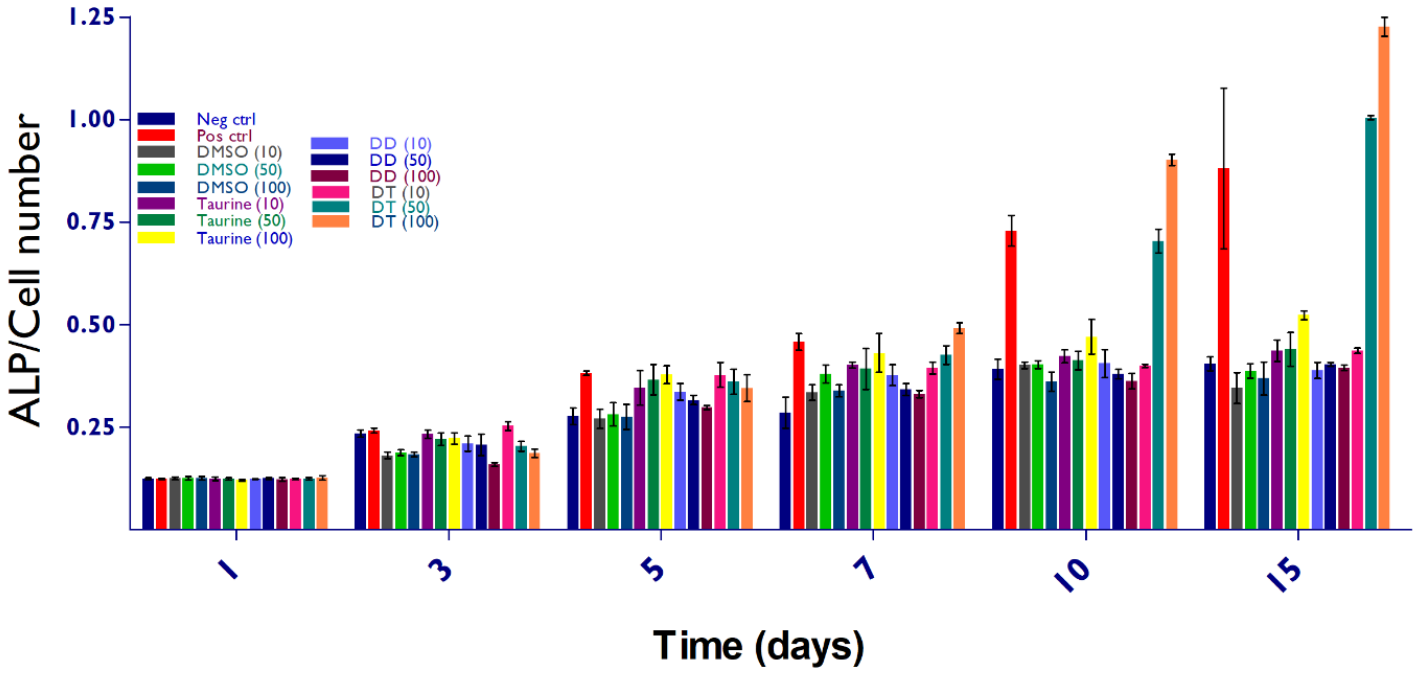



**Figure S2**: Figure shows quantification of ALP activity of murine pre-osteoblasts of MC3T3E1 into osteogenic lineage in a dose-dependent fashion with all the controls taken into consideration for 15 days (Negative control: untreated, DMSO: vehicle solvent, Positive control: osteogenic media). The value in parentheses indicates the concentration of compounds tested in µM.

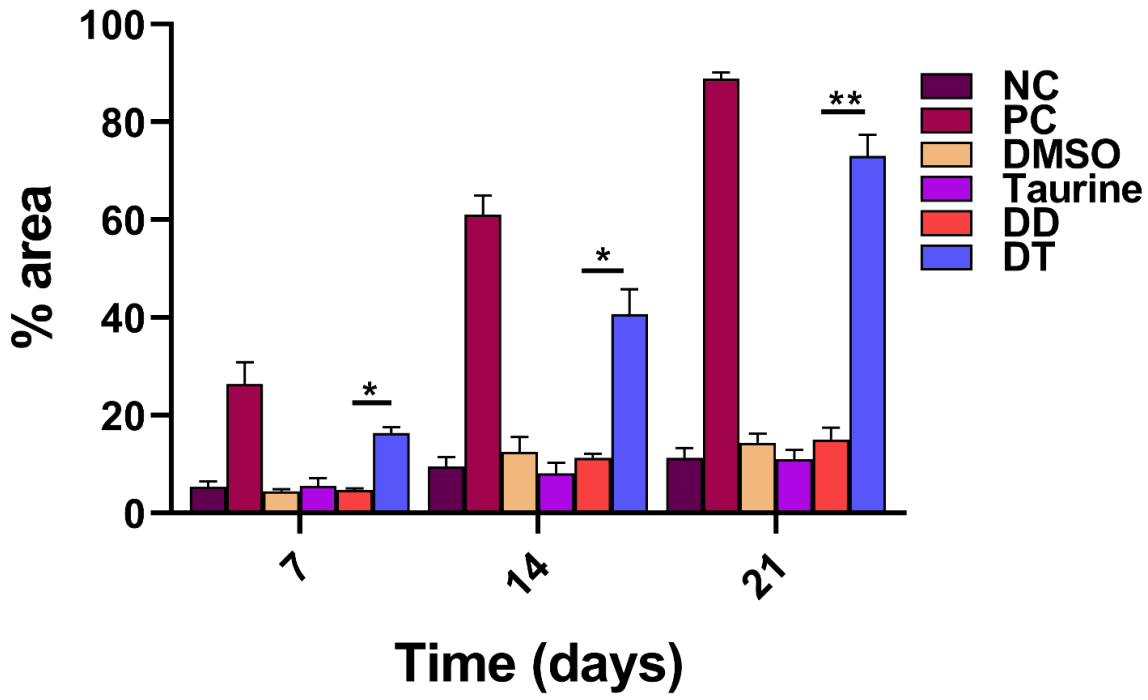



Figure S3: Quantification of alizarin red staining via Image j software by calculation of percentage area under calcium deposition at specific thresholding.

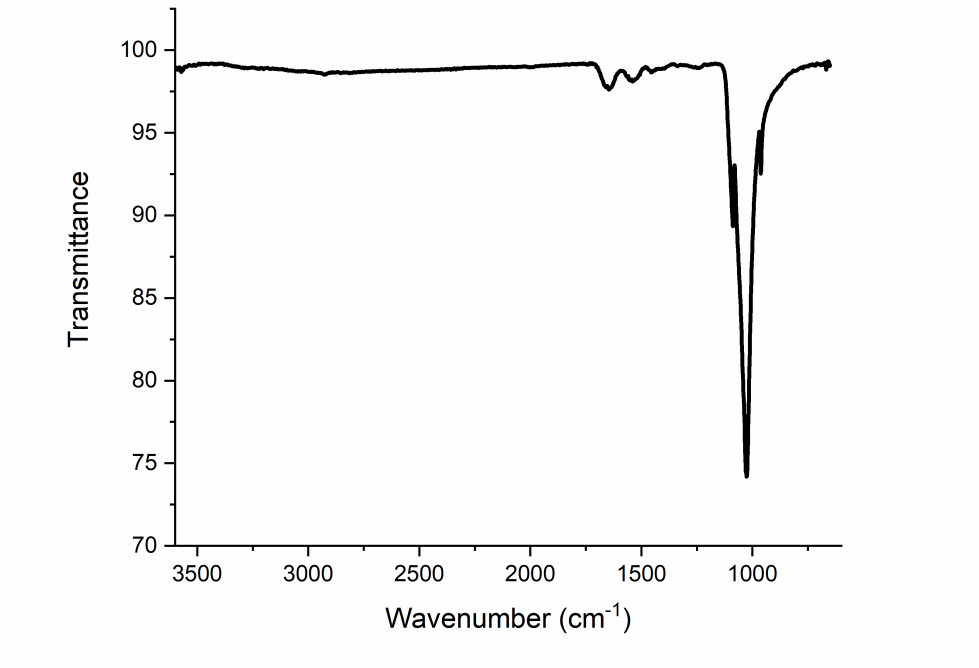



**Figure S4**: FTIR spectra of cryogel composite

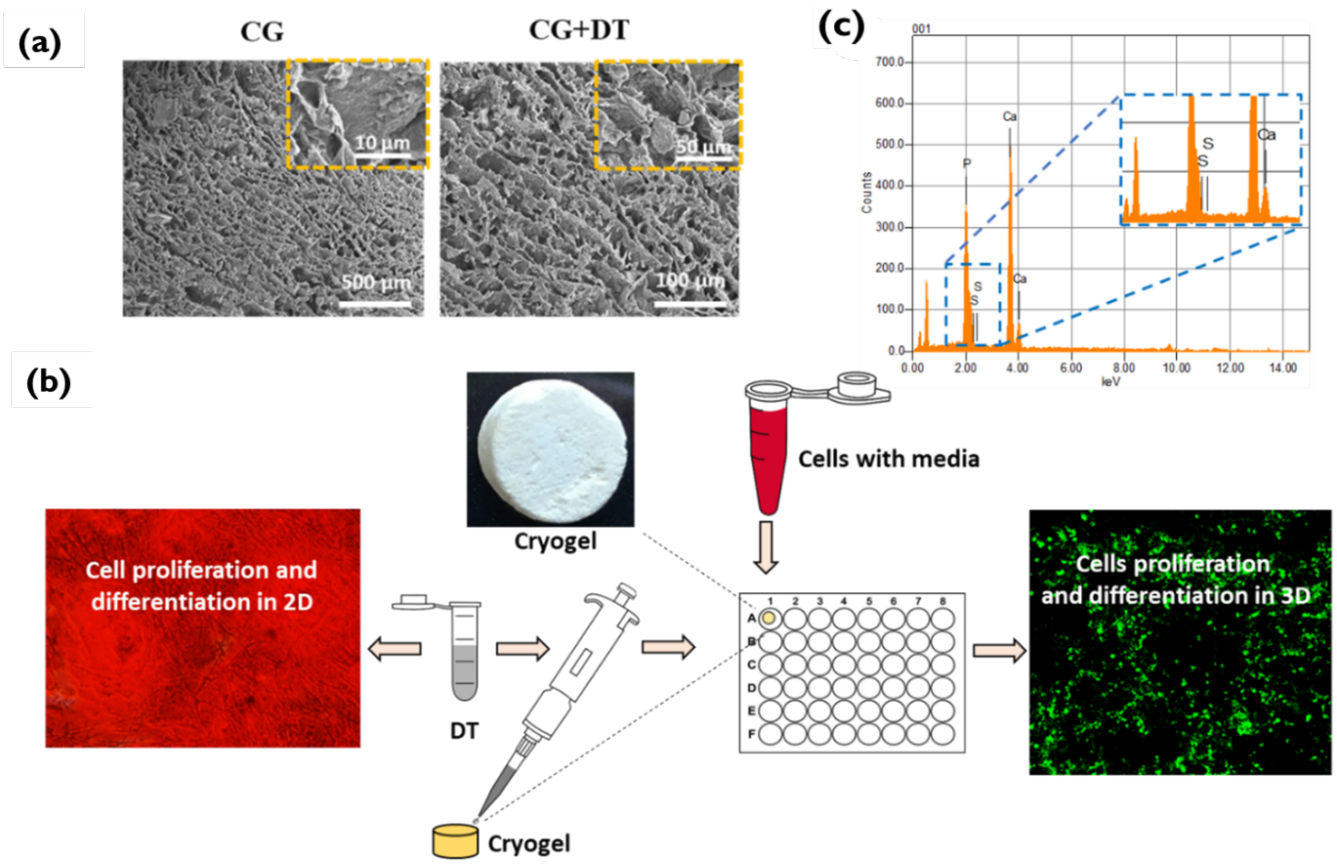



**Figure S5**: (a) SEM images of untreated cryogel and DT treated cryogel (CG - cryogel, CG+DT - DT treated cryogel), (b) Schematic illustration of procedure for exploring osteoinductive effect of DT in 2D and 3D cell culture, (c) EDS spectrum of DT incorporated cryogel.

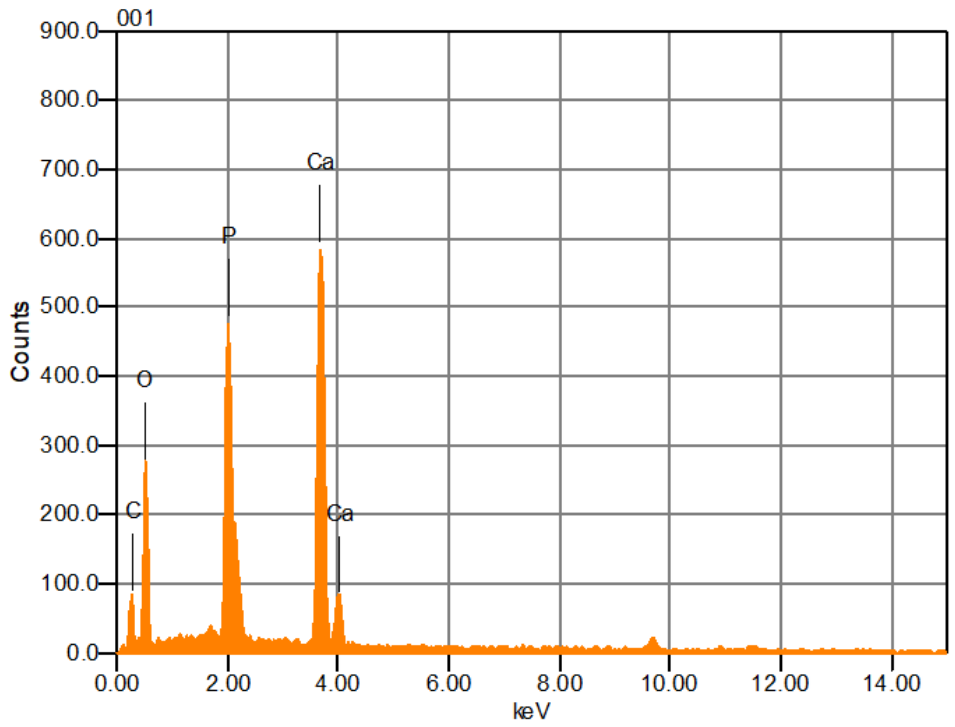



**Figure S6**: EDS spectrum of untreated cryogel.

## 4. HPLC spectra

### (a) DT

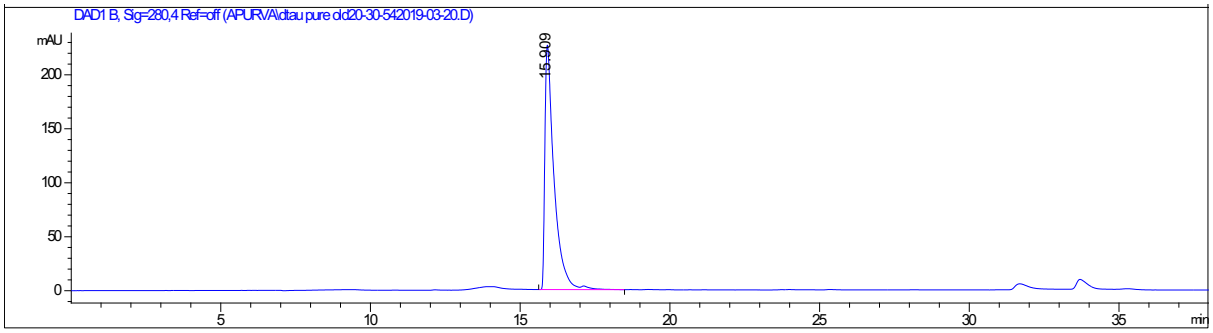



**Figure S7:** HPLC chromatogram for DT in 0.1% TFA- $CH_3CN$ /$H_2O$ gradient system.

**(b) MT**

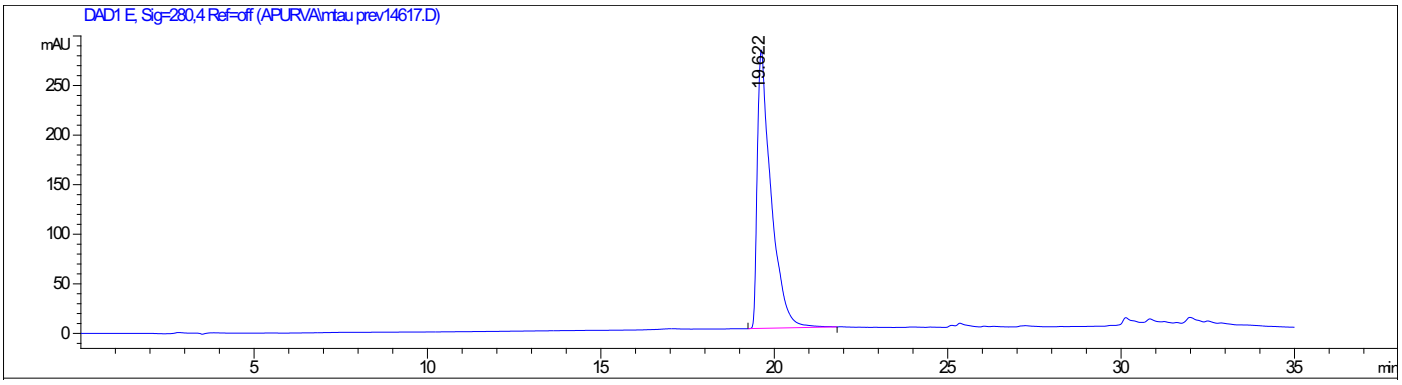



**Figure S8:** HPLC chromatogram for MT in 0.1% TFA- $CH_3CN$ /$H_2O$ gradient system.

**(c) DD**

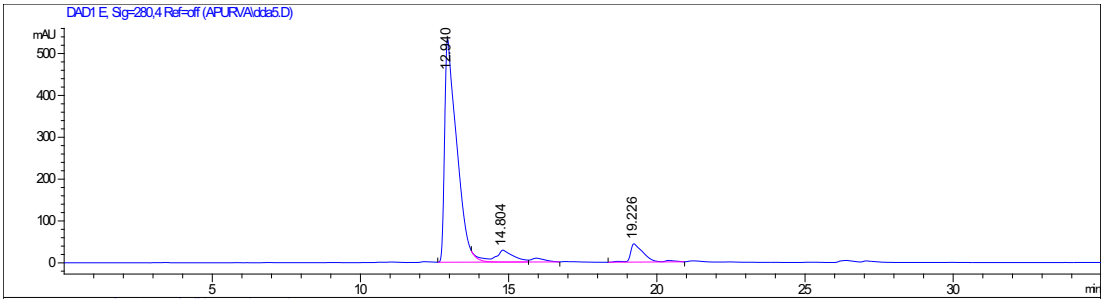
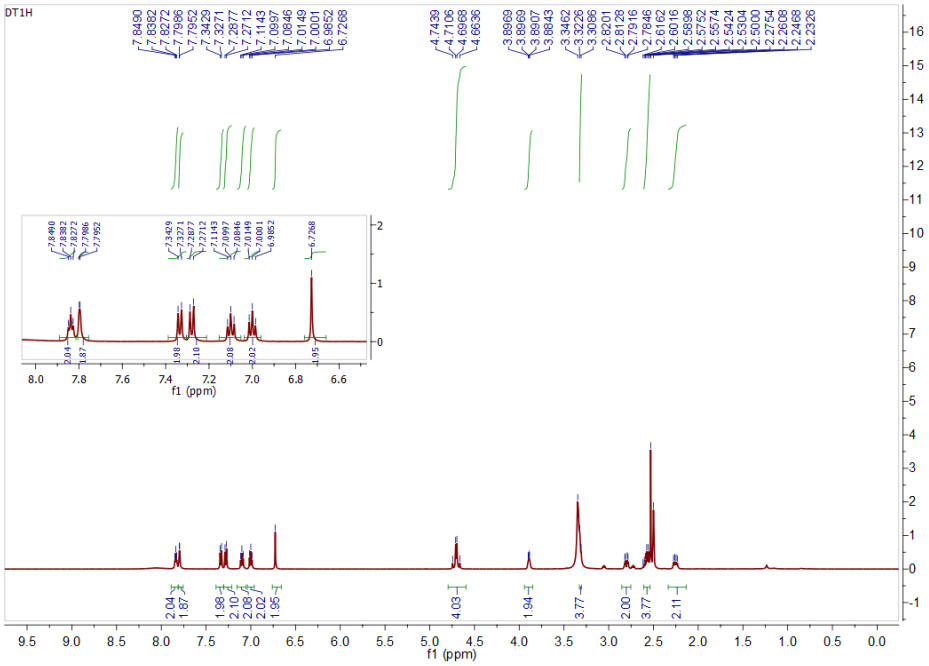
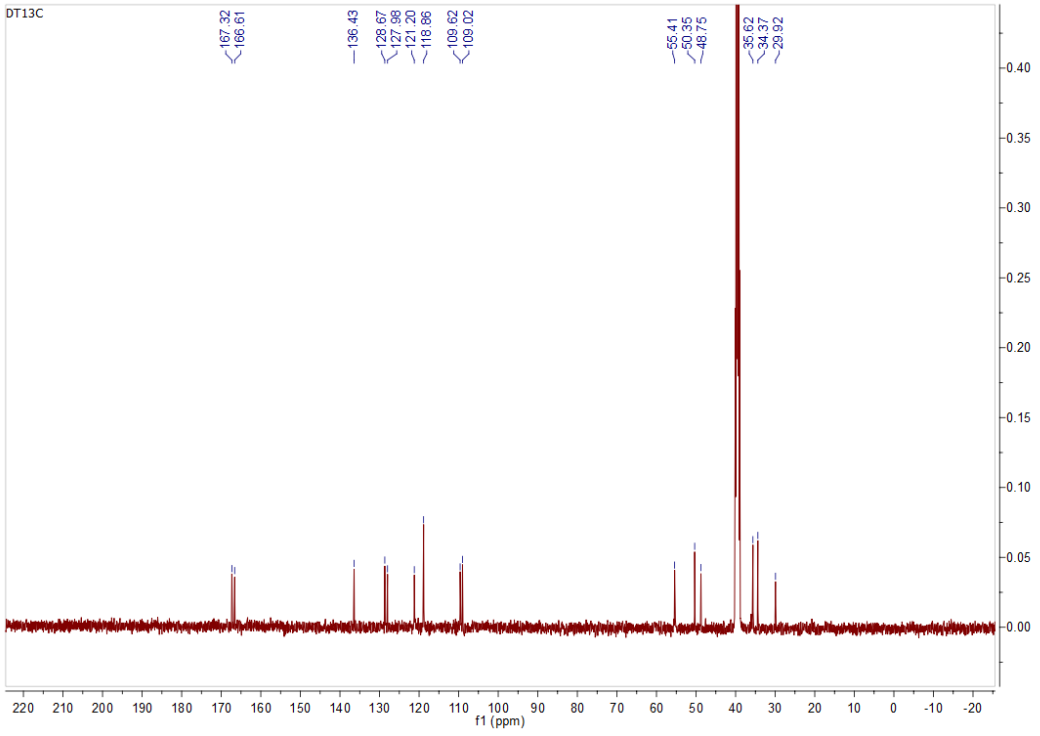



**Figure S9:** HPLC chromatogram for DD in 0.1% TFA- $CH_3CN$ /$H_2O$ gradient system.

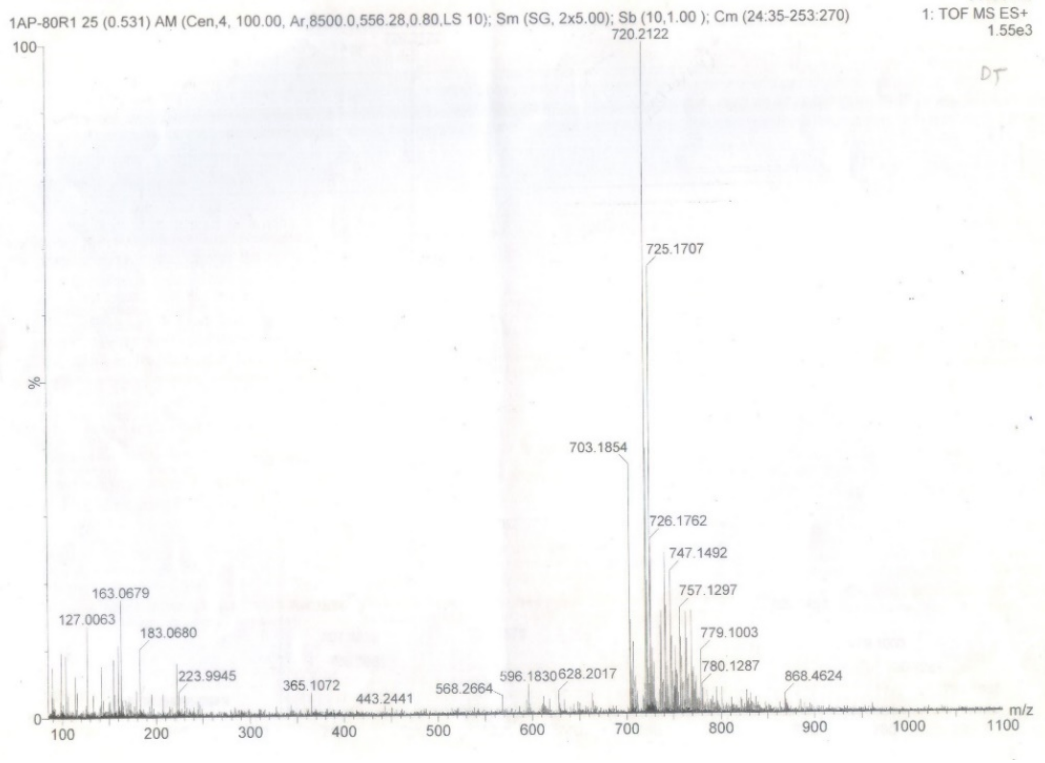
## 5. $^{1}H$ ,$^{13}C$ NMR and ESI-HRMS spectra



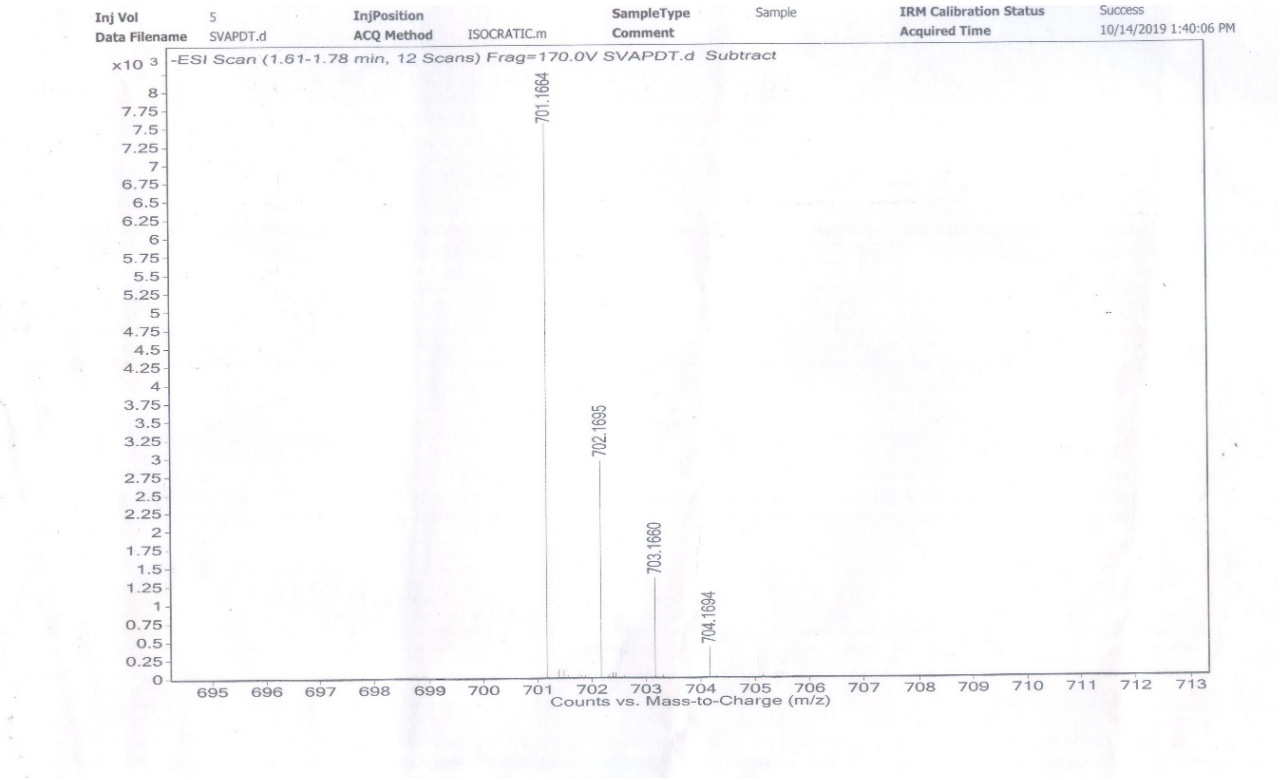

**Spectra 1 :** $^{1}H$ NMR spectra of DT in DMSO-$d_6$




**Spectra 2:** $^{13}C$ NMR spectra of DT in DMSO-$d_6$




**Spectra 3**: ESI-HRMS of DT




**Spectra 4**: ESI-HRMS of DT (negative mode)

References

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(2) Teotia, A. K.; Raina, D. B.; Singh, C.; Sinha, N.; Isaksson, H.; Tägil, M.; Lidgren, L.; Kumar, A. Nano-hydroxyapatite bone substitute functionalized with bone active molecules for enhanced cranial bone regeneration. *ACS Appl. Mater. Interfaces* 2017, *9* (8), 6816-6828.

(3) Teotia, A. K.; Qayoom, I.; Kumar, A. Endogenous Platelet-Rich Plasma Supplements/Augments Growth Factors Delivered via Porous Collagen-Nanohydroxyapatite Bone Substitute for Enhanced Bone Formation. *ACS Biomater. Sci. Eng.* 2019, *5* (1), 56-69.