Supporting Information

A tailored 4G *s*-triazine-based dendrimer vehicle for quercetin endowed with MMP-2/9 inhibition and VEGF downregulation for targeting breast cancer progression and liver metastasis

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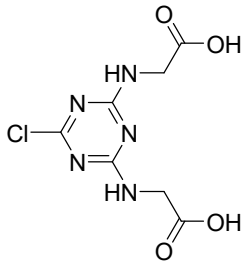
1. Chemistry

1.1. Materials & Methods:

Solvents and all reagents were purchased from Sigma-Aldrich. Unless otherwise stated, normal workup from organic solvent involved drying over Na_2SO_4 and rotary evaporation. TLC was performed using aluminum-backed Merck silica gel 60 F-254 plates using suitable solvent systems, with spots being visualized by a Spectroline UV Lamp (254 or 365 nm) or I_2 vapor. Melting points were obtained in open capillary tubes by using a MEL-Temp II melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a PerkinElmer 1600 series Fourier transform instrument as KBr pellets. The absorption bands ($\bar{\nu}_{max}$) are given in wave numbers (cm^{-1}). Nuclear magnetic resonance (NMR) spectra (^1H NMR and ^{13}C NMR) were recorded on JEOL 500 MHz spectrometers at ambient temperature. Chemical shifts are reported in parts per million (ppm) and are referenced relative to residual solvent (e.g., $CHCl_3$ at δ 7.26 ppm for $CDCl_3$, DMSO at δ 2.50 ppm for DMSO-d_6). Elemental analyses were performed on a PerkinElmer 2400 elemental analyzer, and the values found were within ±0.3% of the theoretical ones.

1.1.1. Preparation of third and fourth generation triazine dendrimers:

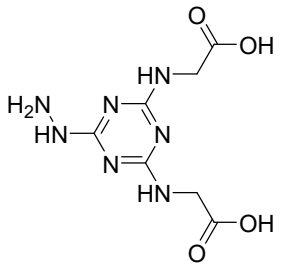
Preparation of 2,2'-((6-chloro-1,3,5-triazine-2,4-diyl)bis(azanediyl))diacetic acid 3 [1]



A solution of cyanuric chloride **1** (1.84 g, 10 mmol) in acetone (10 mL) was added dropwise with stirring to an ice-cooled stirred mixture of glycine **2** (1.50 g, 20 mmol) and Na_2CO_3 (4.24 g, 40 mmol) in water (15 mL). The reaction mixture was stirred overnight, neutralized with hydrochloric acid till complete precipitation, filtered off, washed and recrystallized from acetone.

The product was obtained as pale yellow powder, 2.4 g (91.7%) yield; mp 220-221°C (dec.); IR (KBr): 3650-2400 (br, O-H), 3426, 3258 (N-H), 2986 (sp^3 CH), 1735 (C=O) cm^{-1}; ^1H-NMR (500 MHz, DMSO-d_6): δ 3.82-3.90 (m, 3H, 3 α-CH), 4.01-4.75 (m, 1H, 1 α-CH), 7.89-8.04 (m, 1H, 1 NH, D_2O exchangeable), 8.09-8.40 (m, 1H, 1 NH, D_2O exchangeable), 12.60 (br.s, 2H, 2 COOH, D_2O exchangeable). Elemental analysis: calculated for $C_7H_8ClN_5O_4$: C, 32.14; H, 3.08; N, 26.77. Found: C, 32.20; H, 3.30; N, 26.48.

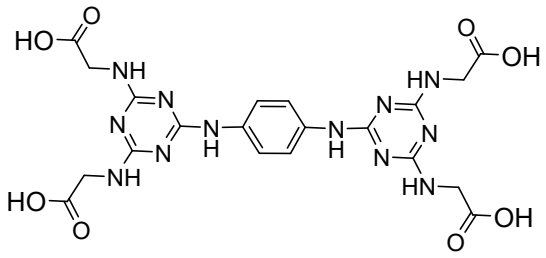
Preparation of 2,2'-((6-hydrazinyl-1,3,5-triazine-2,4-diyl)bis(azanediyl))diacetic acid 4 [2]



Hydrazine hydrate 100% (2 mL, 41 mmol) was added dropwise to a solution of 2,2'-((6-chloro-1,3,5-triazine-2,4-diyl)bis(azanediyl))diacetic acid **3** (0.26 g, 1 mmol) in water (3 mL). The reaction mixture was then stirred overnight. Excess methanol was added until complete precipitation, filtered off, washed and recrystallized from methyl alcohol to afford the desired pure product **4**.

The product was obtained as white powder, 0.23 g (89.4%) yield; mp 269°C (dec.); IR (KBr): 3600-2700 (br, O-H), 3387 (N-H), 2927 (sp^3 CH), 1746 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.83-3.89 (m, 6H, 2 α-CH_2 + 1 NH_2, D_2O exchangeable), 6.15-6.27 (dis.t, 1H, 1 NH-NH_2, D_2O exchangeable), 6.62-6.95 (m, 2H, 2 NH-CH_2, D_2O exchangeable). Elemental analysis: calculated for $C_7H_{11}N_7O_4$: C, 32.69; H, 4.31; N, 38.12. Found: C, 32.75; H, 4.20; N, 38.22.

Preparation of 1st generation acid-terminated dendrimer (TPhyG_1-OH) 6 [3]

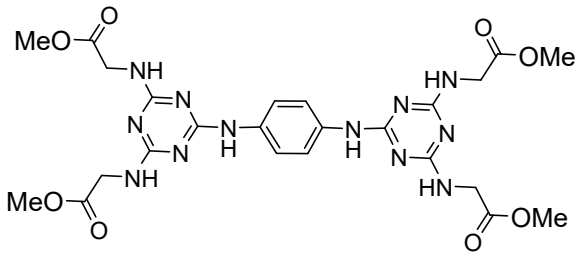


A solution of the *p*-phenylenediamine **5** (0.81 g, 7.5 mmol) in dioxane (5 mL) was added to a stirred mixture of diacetic acid **3** (3.92 g, 15 mmol) and Na_2CO_3 (4.77 g, 45 mmol) in water (10 mL). The reaction mixture was heated under reflux for 10 h. The progress of the reaction was monitored by TLC using methanol: methylene chloride (2: 1) as an eluent. After the completion of the reaction, the reaction mixture was neutralized with

HCl, filtered off and washed with acetone and recrystallized from ethanol to afford the corresponding tetracarboxylic acid.

Compound **6** was obtained as a light violet powder 3.92 g (93.7%) yield; mp over 300°C (dec.) [Lit. >300°C] [3]; IR (KBr): 3650-2400 (br, O-H), 3436 (N-H), 2926, 2856 (sp^3 CH), 1636 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, D_2O): δ 3.80-4.10 (m, 8H, 4 α-CH_2), 7.10-7.30 (m, 4H, Ar-H). Elemental analysis: calculated for $C_{20}H_{22}N_{12}O_8$: C, 43.01; H, 3.97; N, 30.10. Found: C, 43.23; H, 3.81; N, 30.28.

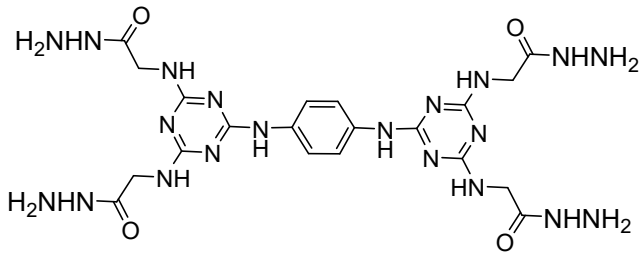
Preparation of 1st generation ester-terminated dendrimer (TPhyG$_1$-OMe) 7 [3]



To a solution of tetracarboxylic acid **6** (2.79 g, 5 mmol) in methanol (30 mL), five drops of conc. sulfuric acid were added. The reaction mixture was refluxed for 10 h. The progress of the reaction was followed by TLC using methanol: methylene chloride (1:2) as an eluent. After the reaction was completed, the reaction mixture was left to cool down to room temperature, neutralized with 10% sodium bicarbonate solution till complete precipitation. The precipitate was filtered off, washed and recrystallized from ethanol to afford the corresponding tetramethyl tetracarboxylate **7** in excellent yield.

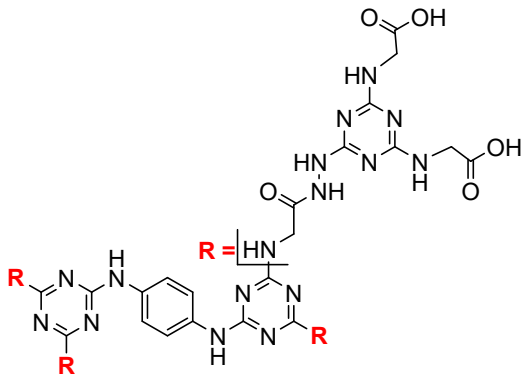
The tetramethyl tetracarboxylate derivative **7** was obtained as a light violet powder, 2.9 g (94.5%) yield; mp 140-142°C (dec.) [Lit. >300°C] [3];; IR (KBr): 3554, 3471, 3345 (N-H), 3095 (sp^2 CH), 2958 (sp^3 CH), 1743 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.58-3.72 (m, 12H, 4 O-CH_3), 3.98-4.21 (m, 8H, 4 α-CH_2), 7.39-7.62 (m, 4H, Ar-H), 8.00-8.75 (m, 4H, 4 NH-CH_2, D_2O exchangeable), 10.01, 10.31 (2s, 2H, 2 NH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 42.40, 42.77, 49.12, 52.42, 52.65, 53.47, 121.98, 122.17, 122.32, 134.40, 161.62, 170.11, 170.22. Elemental analysis: calculated for $C_{24}H_{30}N_{12}O_8$: C, 46.90; H, 4.92; N, 27.35. Found: C, 46.96; H, 4.85; N, 27.46.

Preparation of 1st generation hydrazide-terminated dendrimer (TPhyG$_1$-NH$_2$) 8 [3]



To a solution of the corresponding ester **7** (3.07 g, 5 mmol) in methanol (30 mL), hydrazine hydrate (6 mL) was added. The reaction mixture was heated under reflux for 6 h. The corresponding hydrazide derivative **8** was filtered off and washed with ethanol. Hydrazide derivative **8** was obtained as a light violet powder 2.72 g (88.6%) yield; mp over 300°C (dec.) [Lit. >300°C] [3]; IR (KBr): 3368, 3294 (N-H), 3048 (sp^2 CH), 2949 (sp^3 CH), 1655 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, D_2O): δ 3.80-4.10 (m, 8H, 4 α-CH_2), 7.10-7.30 (m, 4H, Ar-H). Elemental analysis: calculated for $C_{20}H_{30}N_{20}O_4$: C, 39.09; H, 4.92; N, 45.58. Found: C, 39.31; H, 4.83; N, 45.69.

Preparation of 2nd generation acid-terminated dendrimer (TPhyG_2-OH) 9

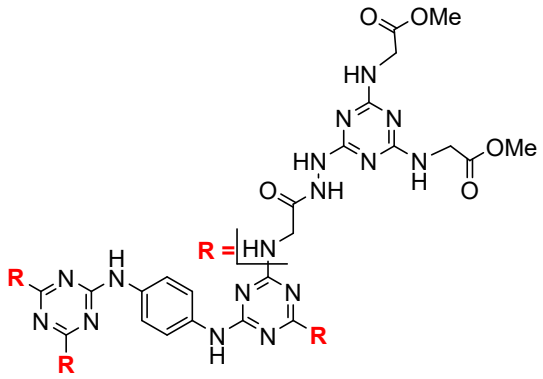


Route i: a mixture of 2,2'-((6-chloro-1,3,5-triazine-2,4-diyl)bis(azanediyl))diacetic acid **3** (1.046 g, 4 mmol) and triethyl amine (1.214 g, 12 mmol) in dioxane/water solvent mixture (1:1) (12 mL) was stirred at room temperature for 1 h. To this mixture, hydrazide derivative **8** (0.614 g, 1 mmol) was added. The reaction mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC using methanol: methylene chloride (2:1) as an eluent. After the completion of the reaction, the desired product was filtered off, washed well with dioxane and recrystallized from ethanol to afford the corresponding 2nd generation dendrimer **9**.

Route ii: to a solution of the prepared ester **7** (0.077 g, 0.125 mmol) in methanol (10 mL), 2,2'-((6-hydrazinyl-1,3,5-triazine-2,4-diyl)bis(azanediyl)) diacetic acid **4** (0.129 g, 0.5 mmol) was added. The reaction mixture was heated under reflux for 12 h. The progress of the reaction was monitored by TLC using methanol: methylene chloride (1:2) as an eluent. The formed precipitate was filtered off and recrystallized from ethanol to afford the corresponding 2nd generation dendrimer **9**.

2nd generation dendrimer **9** was obtained as a light violet powder, **route i** (1.23 g, 81.2% yield); **route ii** (0.126 g, 66.4% yield); mp over 300°C (dec.); IR (KBr): 3600-2780 (br, O-H), 3370, 3295 (N-H), 3020 (sp^2 CH), 2908 (sp^3 CH), 1653 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, D_2O): δ 3.05-3.08 (m, 2H, 1 α-CH_2), 3.32-3.45 (m, 20H, 10 α-CH_2), 3.49-3.52 (m, 2H, 1 α-CH_2), 7.77-7.78 (s, 4H, Ar-H). ^{13}C-NMR (125 MHz, D_2O): δ 39.52, 42.24, 42.74, 116.39, 118.69, 148.26, 156.21, 160.81, 161.10, 161.41, 161.70, 169.22, 169.95, 171.63. Elemental analysis: calculated for $C_{48}H_{58}N_{40}O_{20}$: C, 38.05; H, 3.86; N, 36.98. Found: C, 38.12; H, 3.73; N, 36.75.

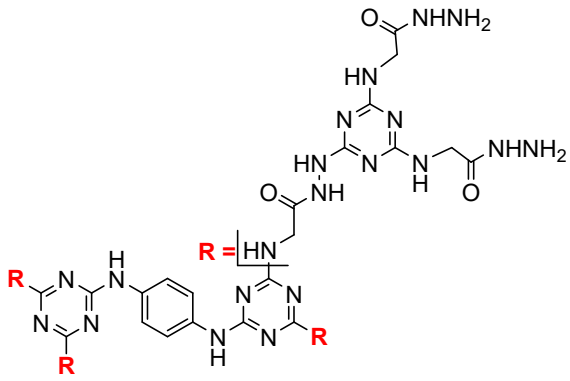
Preparation of 2nd generation ester-terminated dendrimer (TPhyG_2-OMe) 10



To a solution of the 2nd generation octacarboxylic acid dendrimer **9** (4.542 g, 3 mmol) in methanol (30 mL), 5 drops of concentrated sulfuric acid were added drop wise. The reaction mixture was then heated under refluxed for 7 h, the reaction was monitored by TLC using methanol: methylene chloride (1:2) as an eluent. The reaction mixture was left to cool to room temperature and neutralized with 10% sodium bicarbonate solution until complete precipitation. The desired product was filtered off, washed with ethanol and recrystallized from methanol to afford the corresponding pure 2nd generation octamethyl octacarboxylate dendrimer **10**.

Octamethyl octaacetate dendrimer **10** was obtained as a faint violet powder, 4.20 g (86.1%) yield; mp over 300°C (dec.); IR (KBr): 3271 (N-H), 3127 (sp^2 CH), 2954 (sp^3 CH), 1748 (C=O, ester), 1628 (C=O, amide) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.57-3.61 (m, 30H, 8 O-CH_3 + 3 α-CH_2), 3.84-3.90 (m, 12H, 6 α-CH_2), 3.98-4.03 (m, 6H, 3 α-CH_2), 6.70-7.15 (m, 8H, 8 NH, D_2O exchangeable), 7.20-7.95 (m, 16H, 4 Ar-H + 12 NH, D_2O exchangeable), 8.75-9.05 (m, 2H, 2 NH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 41.33, 42.39, 46.66, 52.39, 52.50, 53.74, 128.26, 131.59, 154.02, 155.29, 161.03, 164.34, 164.71, 167.03, 170.73, 171.70, 179.07, 182.69. Elemental analysis: calculated for $C_{56}H_{74}N_{40}O_{20}$: C, 41.33; H, 4.58; N, 34.43. Found: C, 41.25; H, 4.63; N, 34.50.

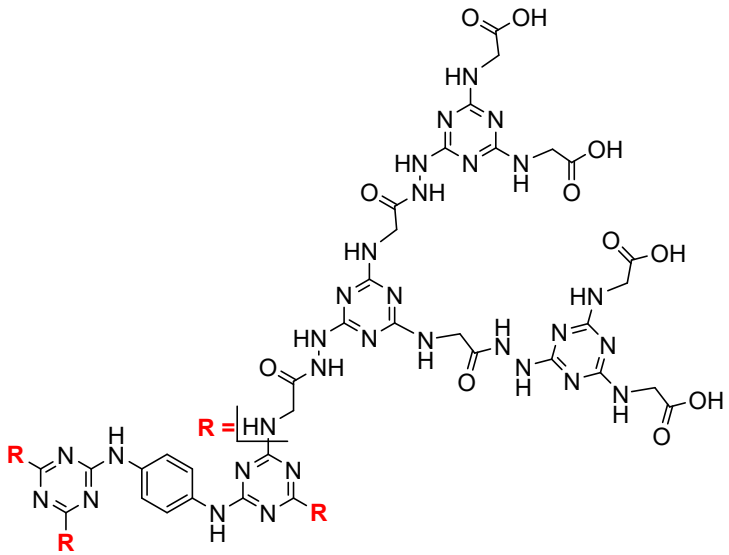
Preparation of 2nd generation hydrazide-terminated dendrimer (TPhyG_2-NH_2) 11



To a solution of octamethyl octaacetate dendrimer **10** (3.252 g, 2 mmol) in methanol (15 mL), hydrazine hydrate (4 mL) was added. The reaction mixture was heated under reflux for 6 h. The reaction mixture was allowed to cool to room temperature until complete precipitation. The precipitate was filtered off, washed with ethanol, and recrystallized from methanol to afford the corresponding octaacetohydrazide dendrimer **11**.

Octaacetohydrazide dendrimer **11** was obtained as a pale violet powder, 2.85 g (87.6%) yield; mp over 300°C (dec.); IR (KBr): 3377, 3294 (N-H), 3183, 3053 (sp^2 CH), 2952 (sp^3CH), 1654 (C=O, amide) cm^{-1}; ^{1}H-NMR (500 MHz, D_2O, TFA): δ 3.31 (br.s, 8H, 4 α-CH_2), 3.35-3.42 (m, 16H, 8 α-CH_2), 6.59-6.65 (m, 4H, Ar-H). ^{13}C-NMR (125 MHz, D_2O, TFA): δ 41.18, 41.55, 113.82, 115.34, 123.63, 123.94, 168.53, 169.90, 171.93 and 172.11. Elemental analysis: calculated for $C_{48}H_{74}N_{56}O_{12}$: C, 35.42; H, 4.58; N, 48.20. Found: C, 35.30; H, 4.39; N, 48.42.

Preparation of 3rd generation acid-terminated dendrimer (TPhyG$_3$-OH) 12



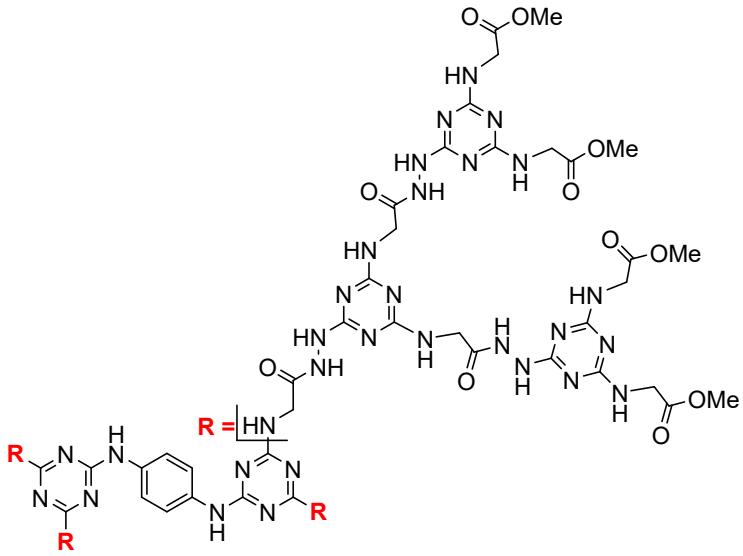
Route i: a mixture of 2,2'-((6-chloro-1,3,5-triazine-2,4-diyl)bis(azanediyl)) diacetic acid **3** (2.092 g, 8 mmol) and triethyl amine (1.619 g, 16 mmol) in dioxane (10 mL) was stirred at room temperature for 1 h. To this mixture, a solution of the previously prepared hydrazide **11** (1.626 gm, 1 mmol) in dioxane/water solvent mixture (1:1) (15 mL) was

added. The reaction mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC using methanol: methylene chloride (2:1) as an eluent. After the completion of the reaction, the desired product was filtered off, washed well with dioxane and recrystallized from ethanol to afford the corresponding 3rd generation dendrimer **12**.

Route ii: to a solution of the prepared ester **10** (3.252 g, 2 mmol) in methanol (30 mL), 2,2'-((6-hydrazinyl-1,3,5-triazine-2,4-diyl)bis(azanediyl)) diacetic acid **4** (4.112 g, 16 mmol) was added. The reaction mixture was heated under reflux for 16 h. The progress of the reaction was monitored by TLC using methanol: methylene chloride (1:2) as an eluent. The formed precipitate was filtered off and washed with methanol and recrystallized from methanol to afford the corresponding 3rd generation dendrimer **12**.

3rd generation dendrimer **12** was obtained as a light brown powder, **route i** (3.05 g, 89%) yield; **route ii** (2.50 g, 73%) yield; mp over 300°C (dec.); IR (KBr): 3514-2500 (O-H), 3384, 3297 (N-H), 3168 (sp^2 CH), 2981, 2931 (sp^3 CH), 1651 (C=O, acid) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.62-3.66 (m, 24H, 12 α-CH_2), 3.83-3.92 (m, 28H, 14 α-CH_2), 4.05-4.06 (m, 4H, 2 α-CH_2), 6.21-6.31 (m, 18H, 18 NH, D_2O exchangeable), 6.63-6.80 (m, 18H, 18 NH, D_2O exchangeable), 6.90-7.20 (m, 6H, 6 NH, D_2O exchangeable), 7.48-7.56 (m, 14H, 4 Ar-H + 10 NH, D_2O exchangeable), 7.96-8.07 (m, 1H, 1 NH, D_2O exchangeable), 8.90 (br.s, 1H, 1 NH, D_2O exchangeable), 10.04-11.96 (br.s, 16H, 16 OH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 41.49, 41.83, 41.93, 42.11, 51.71, 51.95, 155.83, 155.99, 156.02, 165.43, 165.87, 166.14, 170.30, 170.63, 172.16, 172.27. Elemental analysis: calculated for $C_{104}H_{130}N_{96}O_{44}$: C, 36.43; H, 3.82; N, 39.22. Found: C, 36.53; H, 3.99; N, 39.12.

Preparation of 3rd generation ester-terminated dendrimer (TPhyG_3-OMe) 13

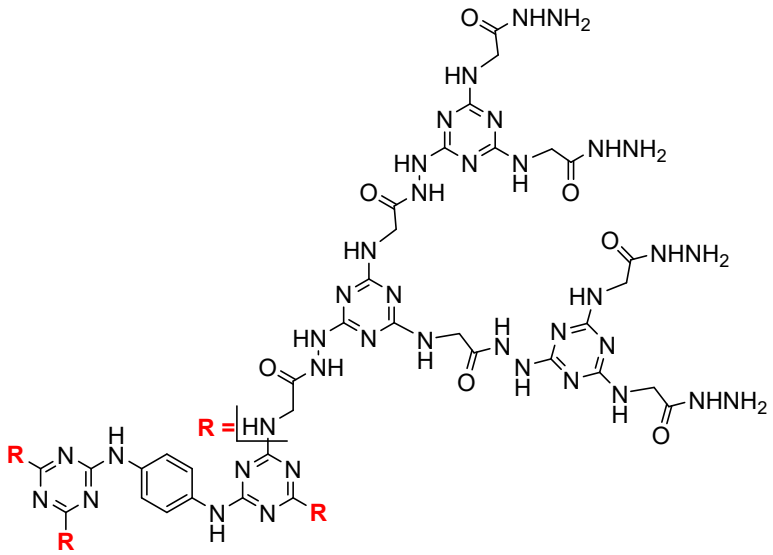


To a solution of the 3rd generation hexadeca-carboxylic acid dendrimer **12** (6.852 g, 2 mmol) in methanol (35 mL), concentrated sulfuric acid (1 mL) was added drop by drop.

The reaction mixture was then refluxed for 7 h, the reaction was monitored by TLC using methanol: methylene chloride (1:2) as an eluent. The reaction mixture was left to cool to room temperature and neutralized with 10% sodium bicarbonate solution until complete precipitation. The desired product was filtered off, washed with ethanol and recrystallized from methanol to afford the corresponding pure 3rd generation hexadecamethyl hexadecacarboxylate dendrimer **13**.

3rd generation hexadecamethyl hexadecacarboxylate dendrimer **13** was obtained as a beige powder, 6.70 g (92%) yield; mp over 300°C (dec.); IR (KBr): 3378, 3276 (N-H), 2954 (sp^3 CH), 1744 (C=O, ester), 1620 (C=O, amide) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.60-3.63 (m, 64H, 16 O-CH_3 + 8 α-CH_2), 3.77-3.98 (m, 40H, 20 α-CH_2), 6.90-7.45 (m, 24H, 24 NH, D_2O exchangeable), 7.46-7.59 (m, 22H, 4 Ar-H + 18 NH, D_2O exchangeable), 8.84-8.89 (m, 12H, 12 NH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 41.83, 42.13, 51.46, 51.62, 119.56, 134.42, 163.65, 163.76, 164.08, 165.52, 165.70, 171.14, 171.29. Elemental analysis: calculated for $C_{120}H_{162}N_{96}O_{44}$: C, 39.45; H, 4.47; N, 36.81. Found: C, 39.30; H, 4.29; N, 36.98.

Preparation of 3rd generation hydrazide-terminated dendrimer (TPhyG$_3$-NH$_2$) 14

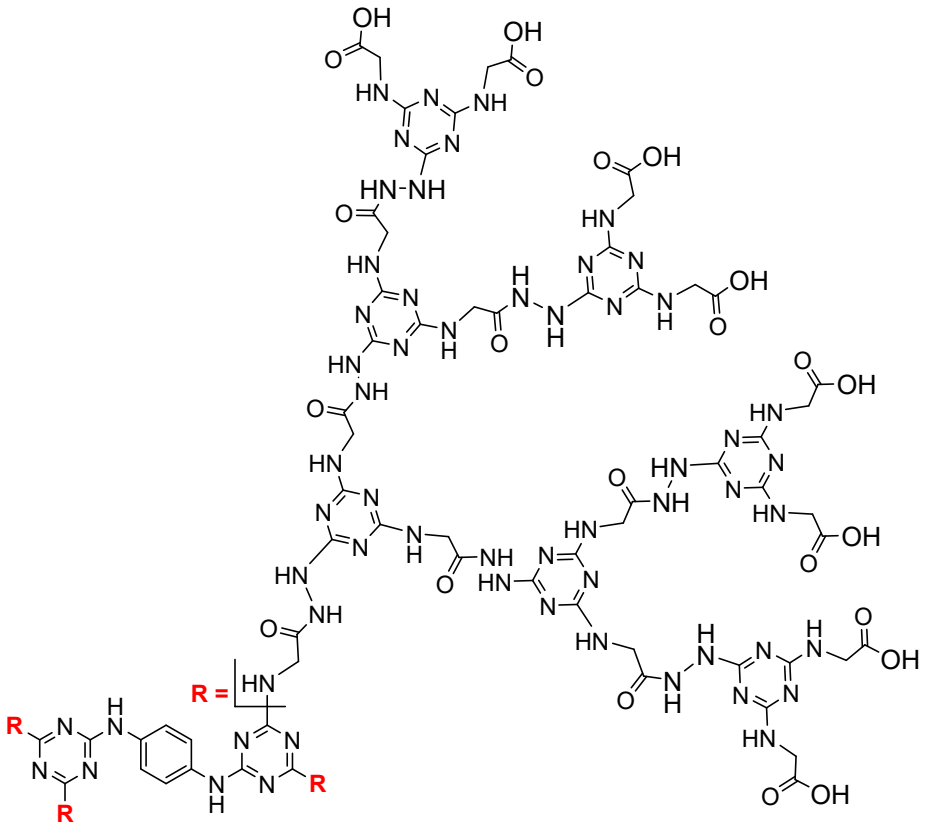


To a solution of hexadecamethyl hexadecaacetate dendrimer **13** (7.30 g, 2 mmol) in methanol (15 mL), hydrazine hydrate (4 mL) was added. The reaction mixture was heated under reflux at 70-80°C for 8 h. The reaction mixture was allowed to cool to room temperature until complete precipitation. The precipitate was filtered off, washed with ethanol, and recrystallized from methanol to afford the corresponding hexadecaacetohydrazide dendrimer **14**.

Hexadecaacetohydrazide dendrimer **14** was obtained as a beige powder, 6.70 g (92%) yield; mp over 300°C (dec.); IR (KBr): 3370, 3294 (N-H), 3046 (sp^2 CH), 2960 (sp^3 CH), 1651 (C=O, amide) cm^{-1}; ^{1}H-NMR (500 MHz, D_2O, TFA): δ 3.61-3.70 (m, 56H, 28 α-CH_2), 6.87-6.97 (m, 4H, Ar-H). ^{13}C-NMR (125 MHz, D_2O, TFA): δ 42.21, 42.33, 42.62,

42.76, 42.85, 123.18, 125.00, 154.25, 155.57, 169.50, 169.63, 169.72, 169.95, 173.13, 173.22, 173.49, 185.39. Elemental analysis: calculated for $C_{104}H_{162}N_{128}O_{28}$: C, 34.19; H, 4.47; N, 49.08. Found: C, 34.09; H, 4.58; N, 49.21.

Preparation of 4th generation acid-terminated dendrimer (TPhyG$_4$-OH) 15



A mixture of 2,2'-((6-chloro-1,3,5-triazine-2,4-diyl)bis(azanediyl))diacetic acid **3** (4.184 g, 16 mmol) and triethyl amine (3.642 g, 36 mmol) in dioxane (20 mL) was stirred at room temperature for 1 h. To this mixture, a solution of hydrazide **14** (3.65 g, 1 mmol) in dioxane/water solvent mixture (1:1) (40 mL) was added. The reaction mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC using methanol/methylene chloride (2:1) as an eluent. After the completion of the reaction, the desired product was filtered off, washed well with dioxane and recrystallized from ethanol to afford the 4th generation dendrimer.

4th generation dendrimer **15** obtained as a light brown powder, 6.30 g (86.9%) yield; mp over 300°C (dec.); IR (KBr): 3550-2600 (O-H), 3328 (N-H), 3168 (sp^2 CH), 2938 (sp^3 CH), 1625 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.69-3.94 (m, 120H, 60 α-CH_2), 6.75-6.97 (m, 36H, 36 NH, D_2O exchangeable), 7.17-7.32 (br.s, 10H, 10 NH, D_2O exchangeable), 7.44-7.69 (m, 28H, 4 Ar-H + 24 NH, D_2O exchangeable), 8.27-8.47 (m, 28H, 28 NH, D_2O exchangeable), 8.72-8.88 (m, 20H, 20 NH, D_2O exchangeable), 10.80-10.90 (m, 32H, 32 OH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 44.28,

47.18, 48.60, 122.53, 124.85, 133.90, 136.23, 165.58, 168.62, 168.98, 172.29, 177.21, 178.15, 178.51, 183.35, 185.69, 188.01. Elemental analysis: calculated for $C_{216}H_{274}N_{208}O_{92}$: C, 35.75; H, 3.81; N, 40.15. Found: C, 35.66; H, 3.56; N, 40.36.

1.1.2. Preparation of PEG-lactobionic acid-quercetin-hexadecacarboxylic acid G_3-triazine dendrimer conjugate (TPhyG_3-OH)-QUR-LA-PEG

Preparation of [quercetin-hexadeca carboxylic acid G_3-triazine dendrimer] conjugate (TPhyG_3-OH)-QUR 16

A mixture of hexadeca-carboxylic acid dendrimer **12** (50 mg, 0.015 mmol) and *N*,*N*-diisopropylethylamine (30.18 mg, 0.234 mmol) in DMSO (3 mL) were *in situ* preactivated by addition of DIC (*N,N'*-diisopropylcarbodiimide) (30.29 mg, 0.234 mmol) and oxyma (33.16 mg, 0.234 mmol) with stirring for 5 minutes at room temperature. Subsequently, quercetin (35.29 mg, 0.117 mmol) was added to the preactivated dendrimer. The reaction mixture was stirred for 24 h at room temperature, then dialyzed against 50% ethanol/water for 24 h [dialysis cassettes (Thermo Scientific™ Slide-A-Lyzer™ 3.5K MWCO, Fischer, England)]. The free unconjugated quercetin was determined using HPLC as detailed in the Supporting Information (see **S19**). While the resulting solution of (TPhyG_3-OH)-QUR conjugate was further purified by dialysis against deionized water/ethanol solvent gradient for 48 h. The purified dialyzed yellow solution was lyophilized to obtain dry yellow powder of (TPhyG_3-OH)-QUR conjugate; mp over 300°C; IR (KBr): 3600-2800 (O-H), 3417 (N-H), 2926 (sp^3 CH), 1647 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.62-4.14 (m, 56H, 28 α-CH_2), 6.13-6.20 (m, 21H, 18 NH + 3 Ar-H_{QUR}), 6.35-6.43 (m, 21H, 18 NH + 3 Ar-H_{QUR}), 6.83-6.87 (m, 9H, 6 NH + 3 Ar-H_{QUR}), 7.42-7.85 (m, 20H, 4 Ar-H + 10 NH + 6 Ar-H_{QUR}), 8.20-8.58 (m, 1H, 1 NH), 8.70-9.02 (m, 2H, 1 NH + 1 OH_{QUR}), 9.25-9.51 (m, 8H, 8 OH_{QUR}), 9.76-10.08 (m, 12H, 12 OH), 10.78-10.92 (m, 1H, 1 OH), 12.32-12.68 (m, 3H, 3 OH_{QUR}).

Preparation of lactobionic acid-quercetin-hexadeca carboxylic acid G_3-triazine dendrimer conjugate (TPhyG_3-OH)-QUR-LA 17

The solution of (TPhyG_3-OH)-QUR conjugate in DMSO (3 mL) was *in situ* preactivated by addition of DIC (14.73 mg, 0.117 mmol) and oxyma (16.58 mg, 0.117 mmol) with stirring for 5 minutes at room temperature. To the preactivated conjugate (TPhyG_3-OH)-QUR, lactobionic acid (20.92 mg, 0.058 mmol) was added. The reaction mixture was stirred for 24 h at room temperature, then dialyzed against 50% ethanol/water for 24 h [dialysis cassettes (Thermo Scientific™ Slide-A-Lyzer™ 3.5K MWCO, Fischer, England)]. The free unconjugated quercetin was determined using HPLC. While, the resulting solution of (TPhyG_3-OH)-QUR-LA conjugate nanoparticles were further purified by dialysis against deionized water/ethanol solvent gradient for 48 h. The purified dialyzed red solution was lyophilized to obtain dry red powder of (TPhyG_3-OH)-QUR-LA conjugate nanoparticle; mp over 300°C; IR (KBr): 3600- 2800(O-H), 3429 (N-H), 2933 (sp^3 CH), 1623 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.40-3.56 (m,

lactobionic protons), 3.63-3.65 (m, 24H, 12 α-CH_2), 3.89-3.92 (m, 28H, 14 α-CH_2), 4.00-4.06 (m, 4H, 2 α-CH_2), 4.11-4.78 (m, lactobionic protons), 5.12-5.15 (m, 3H, lactobionic anomeric protons), 6.13-6.20 (m, 21H, 18 NH + 3 Ar-H_{QUR}), 6.35-6.47 (m, 21H, 18 NH + 3 Ar-H_{QUR}), 6.82-6.87 (m, 9H, 6 NH + 3 Ar-H_{QUR}), 7.41-7.77 (m, 20H, 4 Ar-H + 10 NH + 6 Ar-H_{QUR}), 7.99-8.06 (m, 1H, 1 NH), 8.67-8.99 (br.s, 2H, 1 NH + 1 OH_{QUR}), 9.28-9.40 (m, 6H, 6 OH_{QUR}), 9.56-9.64 (m, 2H, 2 OH_{QUR}), 9.75-10.37 (m, 9H, 9 OH), 10.71-10.92 (m, 1H, 1 OH), 11.20-11.34 (m, 3H, 3 lactobionic carboxylic OH), 12.41-12.54 (m, 3H, 3 OH_{QUR}).

Preparation of PEG-lactobionic acid-quercetin-hexadeca carboxylic acid G_3-triazine dendrimer conjugate (TPhyG_3-OH)-QUR-LA-PEG 18

A solution of (TPhyG_3-OH)-QUR-LA in DMSO (3 mL) was *in situ* preactivated *via* addition of DIC (36.60 mg, 0.292 mmol) and oxyma (41.45 mg, 0.292 mmol) with stirring for 5 minutes at room temperature. To the preactivated (TPhyG_3-OH)-QUR-LA, PEG (488.91 mg, 0.146 mmol) was added. The reaction mixture was stirred for 24 h at room temperature then dialyzed against 50% ethanol/water for 24 h [dialysis tubes (Visking®, 21 mm, MWCO 12000-14000, Serva, USA)]. The free unconjugated quercetin was determined using HPLC. While, the resulting solution of (TPhyG_3-OH)-QUR-LA-PEG conjugate nanoparticles were further purified by dialysis against deionized water/ethanol solvent gradient for 48 h. The purified dialyzed solution was lyophilized to obtain dry orange powder of (TPhyG_3-OH)-QUR-LA-PEG conjugate nanoparticle; mp over 300°C; IR (KBr): 3500-2600 (O-H), 3432 (N-H), 2920 (sp^3 CH), 1637 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.43-3.54 (m, lactobionic protons + PEG polymer backbone), 3.54-4.00 (m, 56H, 28 α-CH_2), 4.54-4.74 (m, lactobionic protons + PEG-OH), 5.09-5.16 (br.s, 3H, lactobionic anomeric protons), 6.12-6.21 (m, 21H, 18 NH + 3 Ar-H_{QUR}), 6.32-6.46 (m, 21H, 18 NH + 3 Ar-H_{QUR}), 6.82-6.88 (m, 9H, 6 NH + 3 Ar-H_{QUR}), 7.38-7.78 (m, 20H, 4 Ar-H + 10 NH + 6 Ar-H_{QUR}), 7.85-8.06 (m, 1H, 1 NH), 8.65-8.95 (m, 2H, 1 NH + 1 OH_{QUR}), 9.16-9.47 (m, 6H, 6 OH_{QUR}), 9.55-9.71 (m, 2H, 2 OH_{QUR}), 9.77-10.38 (m, 8H, 8 OH), 10.77-11.01 (m, 1H, 1 OH), 12.42-12.56 (m, 3H, 3 OH_{QUR}).

1.1.3. Preparation of PEG-lactobionic acid-quercetin-dotriacontacarboxylic acid G_4-triazine dendrimer conjugate (TPhyG_4-OH)-QUR-LA-PEG

Preparation of [quercetin-dotriacontacarboxylic acid G_4-triazine dendrimer] conjugate (TPhyG_4-OH)-QUR 19

Dotriacontacarboxylic acid dendrimer **15**, (50 mg, 0.0069 mmol) and *N*,*N*-diisopropylethylamine (28.50 mg, 0.221 mmol) in DMSO (3 mL) were *in situ* preactivated by addition of DIC (27.83 mg, 0.221 mmol) and oxyma (31.31 mg, 0.221 mmol) with stirring for 5 minutes at room temperature. Subsequently, quercetin (33.32 mg, 0.110 mmol) was added to the preactivated dendrimer. The reaction mixture was stirred for 24 h at room temperature, then dialyzed against 50% ethanol/water for 24 h [dialysis cassettes (Thermo Scientific™ Slide-A-Lyzer™ 3.5K MWCO, Fischer,

England)]. The free unconjugated quercetin was determined using HPLC. While, the resulting solution of (TPhyG_4-OH)-QUR conjugate was further purified by dialysis against deionized water/ethanol solvent gradient for 48 h. The purified dialyzed solution was lyophilized to obtain dry orange powder of Phy-(TG-OH)$_4$-QUR conjugate; mp over 300°C; IR (KBr): 3600-2800 (O-H), 3432 (N-H), 1631 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.82-3.92 (m, 90H, 45 α-CH_2), 4.06-4.11 (m, 30H, 15 α-CH_2), 6.16-6.24 (m, 5H, 5 Ar-H_{QUR}), 6.38-6.44 (m, 5H, 5 Ar-H_{QUR}), 6.73-7.14 (m, 41H, 36 NH + 5 Ar-H_{QUR}), 7.18-7.36 (m, 10H, 10 NH), 7.46-7.86 (m, 38H, 4 Ar-H + 24 NH + 10 Ar-H_{QUR}), 8.72-9.11 (m, 28H, 28 NH), 9.21-9.59 (m, 28H, 20 NH + 8 OH_{QUR}), 9.81-10.01 (m, 7H, 7 OH_{QUR}), 10.82-10.92 (m, 27H, 27 OH), 12.46-12.57 (m, 5H, 5 OH_{QUR}).

Preparation of lactobionic acid-quercetin-dotriacontacarboxylic acid G_4-triazine dendrimer conjugate (TPhyG_4-OH)-QUR-LA 20

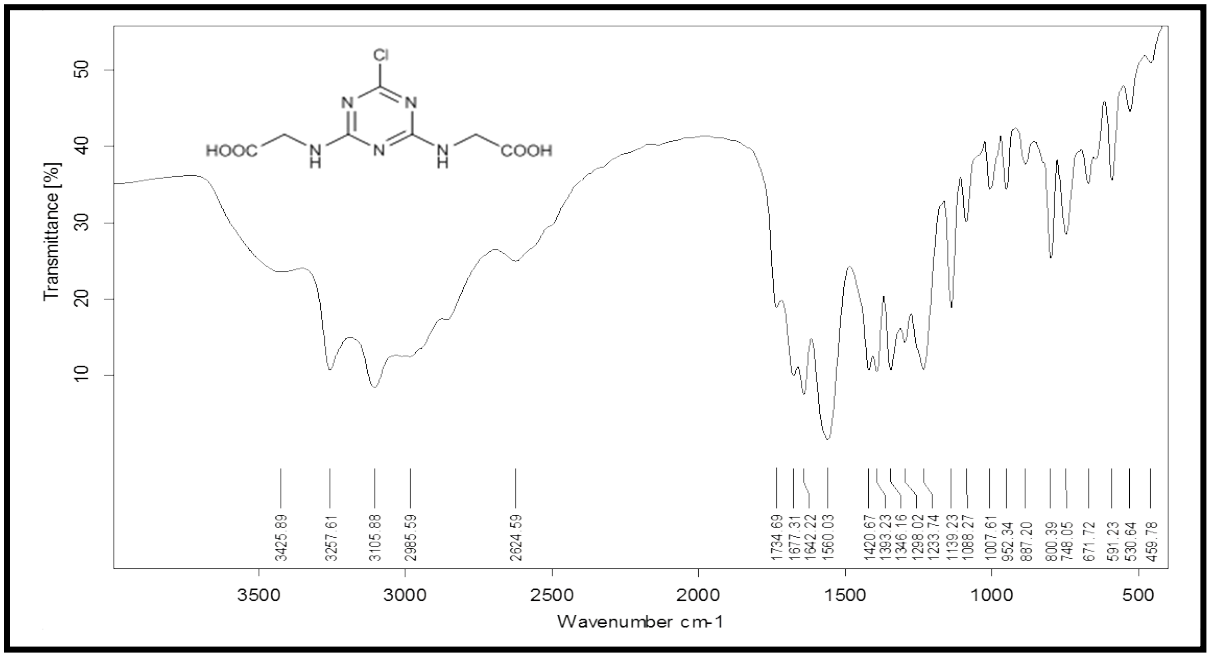
A solution of (TPhyG_4-OH)-QUR conjugate in DMSO (3 mL) was *in situ* preactivated by addition of DIC (13.91 mg, 0.110 mmol) and oxyma (15.66 mg, 0.110 mmol) with stirring for 5 minutes at room temperature. To the preactivated conjugate (TPhyG_4-OH)-QUR, lactobionic acid (19.75 mg, 0.055 mmol) was added. The reaction mixture was stirred for 24 h at room temperature, then dialyzed against 50% ethanol/water for 24 h [dialysis cassettes (Thermo Scientific™ Slide-A-Lyzer™ 3.5K MWCO, Fischer, England)]. The free unconjugated quercetin was determined using HPLC. While the resulting solution of (TPhyG_4-OH)-QUR-LA conjugate nanoparticles were further purified by dialysis against deionized water/ethanol solvent gradient for 48 h. The purified dialyzed orange solution was lyophilized to obtain dry orange powder of (TPhyG_4-OH)-QUR-LA conjugate nanoparticle; mp over 300°C; IR (KBr): 3600-2800 (O-H), 3435 (N-H), 2928 (sp^3 CH), 1628 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.40-3.69 (m, lactobionic protons), 3.93-3.98 (m, 44H, 22 α-CH_2), 4.06-4.12 (m, 76H, 38 α-CH_2), 4.19-4.25, 4.46-4.51 (m, lactobionic protons), 5.17-5.28 (br.s, 7H, lactobionic anomeric protons), 6.17-6.31 (m, 5H, 5 Ar-H_{QUR}), 6.37-6.46 (m, 5H, 5 Ar-H_{QUR}), 6.85-7.21 (m, 41H, 36 NH + 5 Ar-H_{QUR}), 7.32-7.42 (m, 10H, 10 NH), 7.48-8.02 (m, 38H, 4 Ar-H + 24 NH + 10 Ar-H_{QUR}), 8.74-9.13 (m, 28H, 28 NH), 9.31-9.58 (m, 28H, 20 NH + 8 OH_{QUR}), 9.84-10.09 (m, 7H, 7 OH_{QUR}), 10.81-11.16 (m, 27H, 20 OH + 7 lactobionic carboxylic OH), 12.49-12.60 (br.s, 5H, 5 OH_{QUR}).

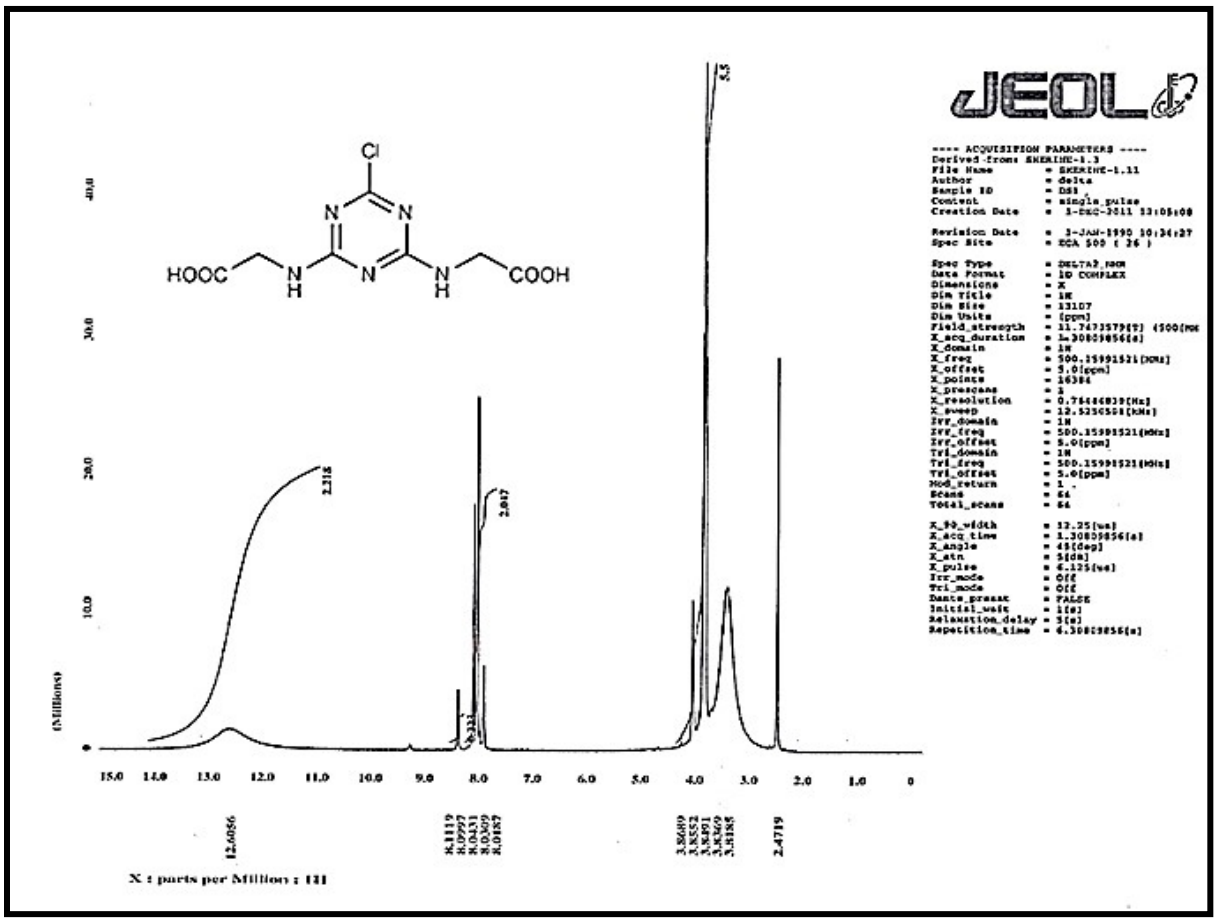
Preparation of PEG-lactobionic acid-quercetin-dotriacontacarboxylic acid G_4-triazine dendrimer conjugate (TPhyG_4-OH)-QUR-LA-PEG 21

A solution of (TPhyG_4-OH)-QUR-LA in DMSO (3 mL) was *in situ* preactivated *via* addition of DIC (17.42 mg, 0.138 mmol) and oxyma (19.57 mg, 0.138 mmol) with stirring for 5 minutes at room temperature. To the preactivated (TPhyG_4-OH)-QUR-LA, PEG (230.85 mg, 0.069 mmol) was added. The reaction mixture was stirred for 24 h at room temperature then dialyzed against 50% ethanol/water for 24 h [dialysis tubes

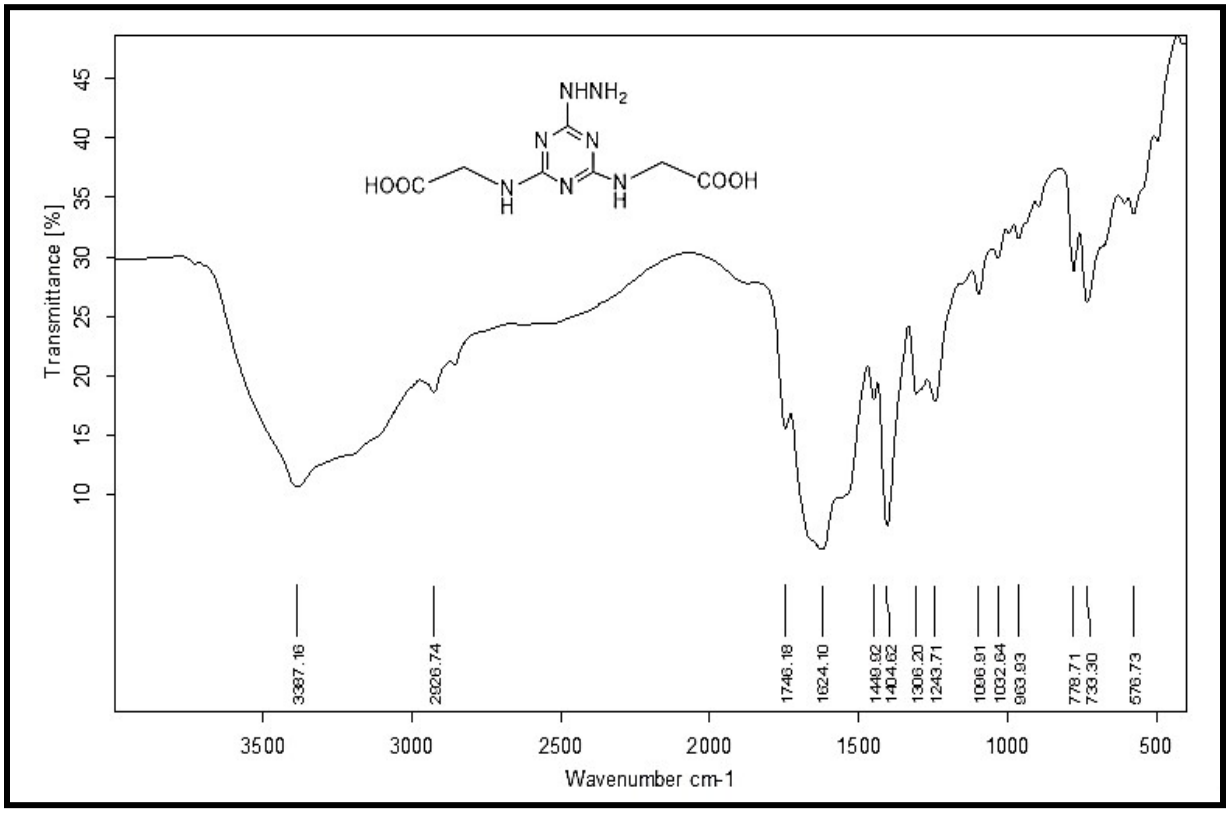
(Visking®, 21 mm, MWCO 12000-14000, Serva, USA)]. The free unconjugated quercetin was determined using HPLC. While, the resulting solution of (TPhyG$_4$-OH)-QUR-LA-PEG conjugate nanoparticles were further purified by dialysis against deionized water/ethanol solvent gradient for 48 h. The purified dialyzed solution was lyophilized to obtain dry orange powder of (TPhyG$_4$-OH)-QUR-LA-PEG conjugate nanoparticle; mp over 300°C; IR (KBr): 3550-2700 (O-H), 3431 (N-H), 2918 (sp^3 CH), 1631 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.40-3.53 (m, lactobionic protons + PEG polymer backbone), 3.53-4.06 (m, 120H, 60 α-CH_2), 4.59-4.63 (m, lactobionic protons + PEG-OH), 5.16-5.21 (br.s, 7H, lactobionic anomeric protons), 6.15-6.23 (m, 5H, 5 Ar-H$_{QUR}$), 6.38-6.44 (m, 5H, 5 Ar-H$_{QUR}$), 6.83-7.18 (m, 41H, 36 NH + 5 Ar-H$_{QUR}$), 7.23-7.33 (m, 10H, 10 NH), 7.47-7.90 (m, 38H, 4 Ar-H + 24 NH + 10 Ar-H$_{QUR}$), 8.73-9.07 (m, 28H, 28 NH), 9.26-9.57 (m, 28H, 20 NH + 8 OH$_{QUR}$), 9.69-9.95 (m, 7H, 7 OH$_{QUR}$), 10.76-11.22 (m, 26H, 19 OH + 7 lactobionic carboxylic OH), 12.45-12.59 (br.s, 5H, 5 OH$_{QUR}$).

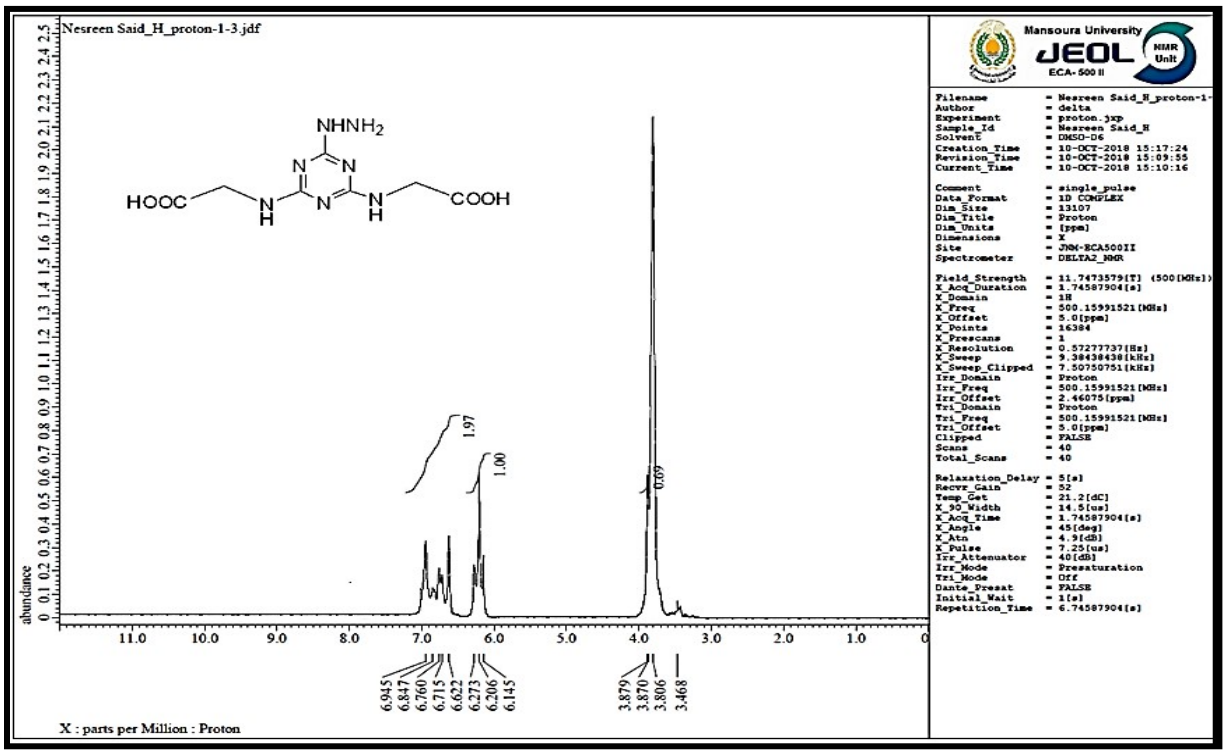
S1. IR (KBr) and ^{1}H-NMR (DMSO-d_6) of Compound **3**



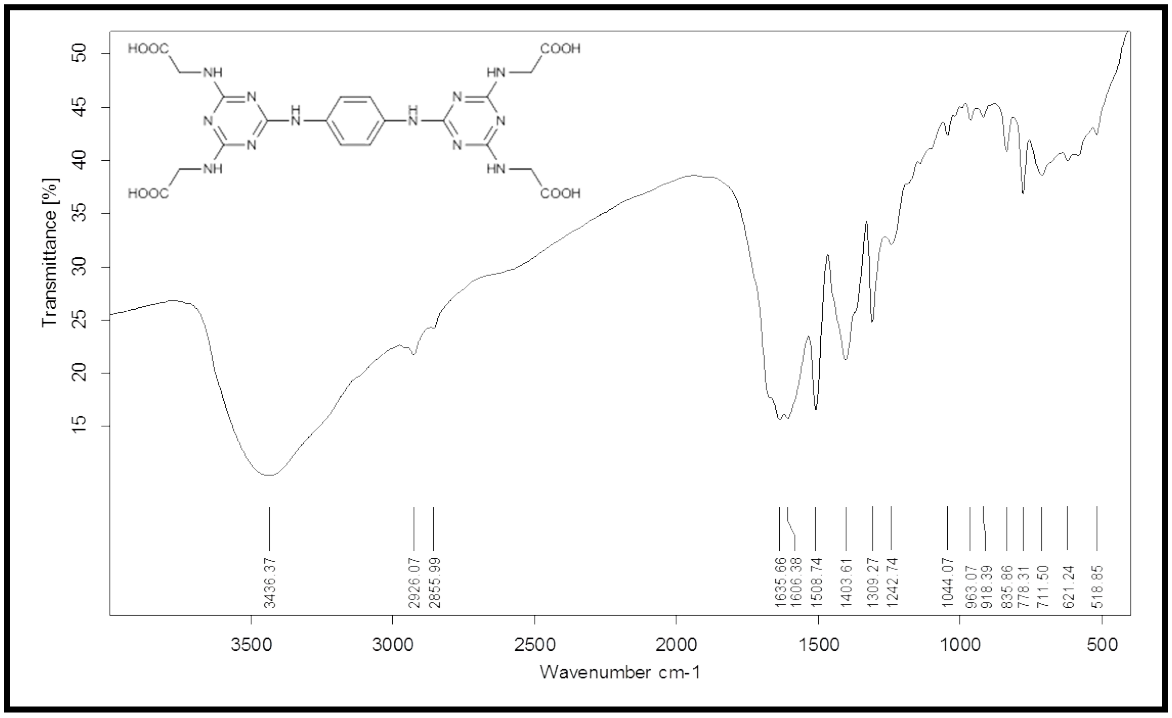


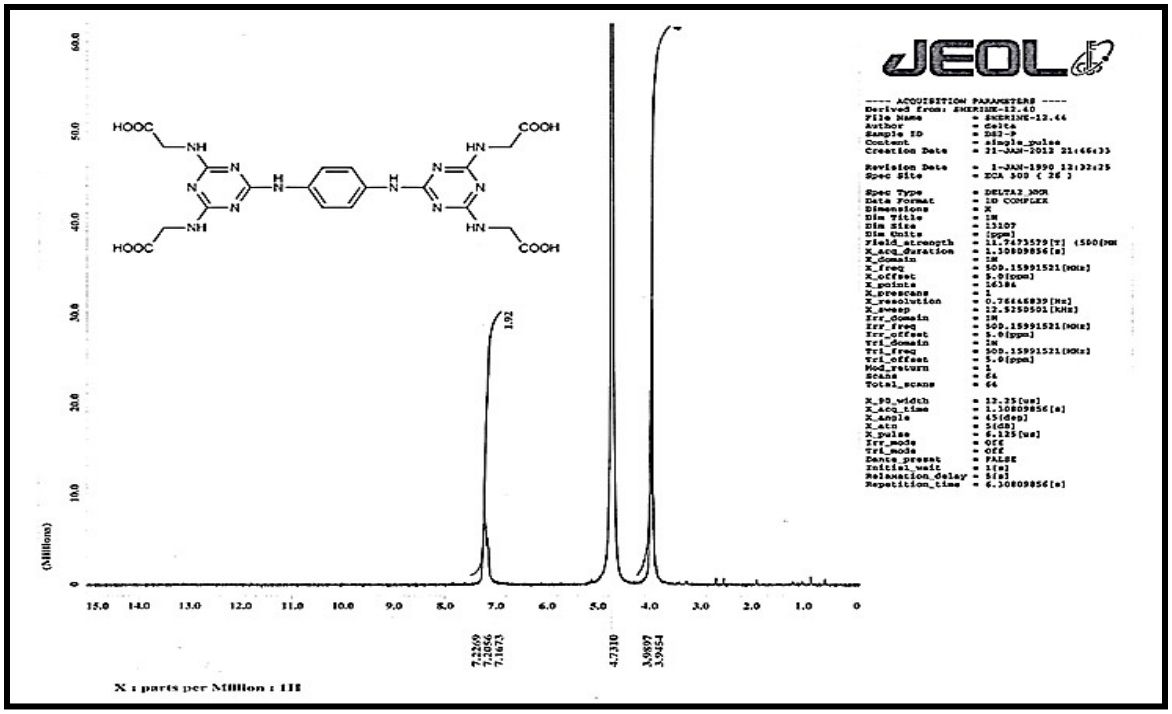
S2- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of **4**



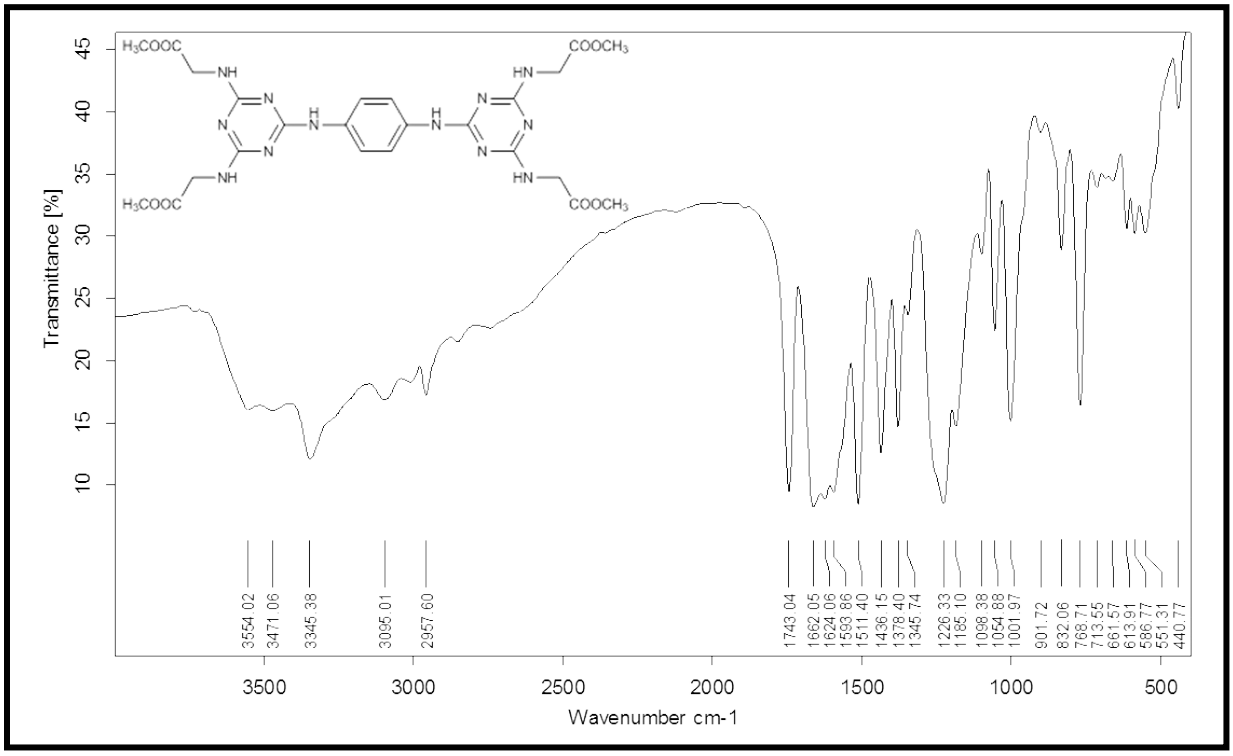


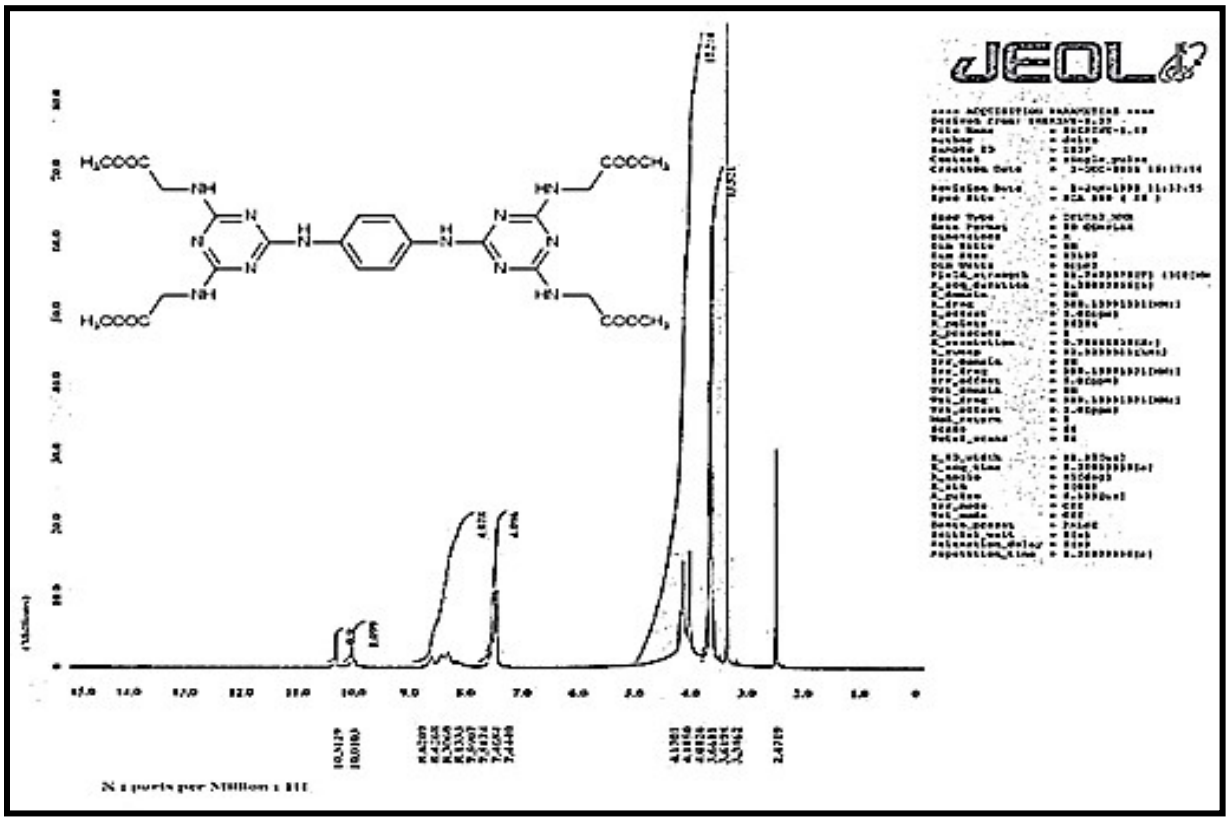
S3- IR (KBr) and ^{1}H-NMR (D_2O) of (TPhyG_1-OH) **6**

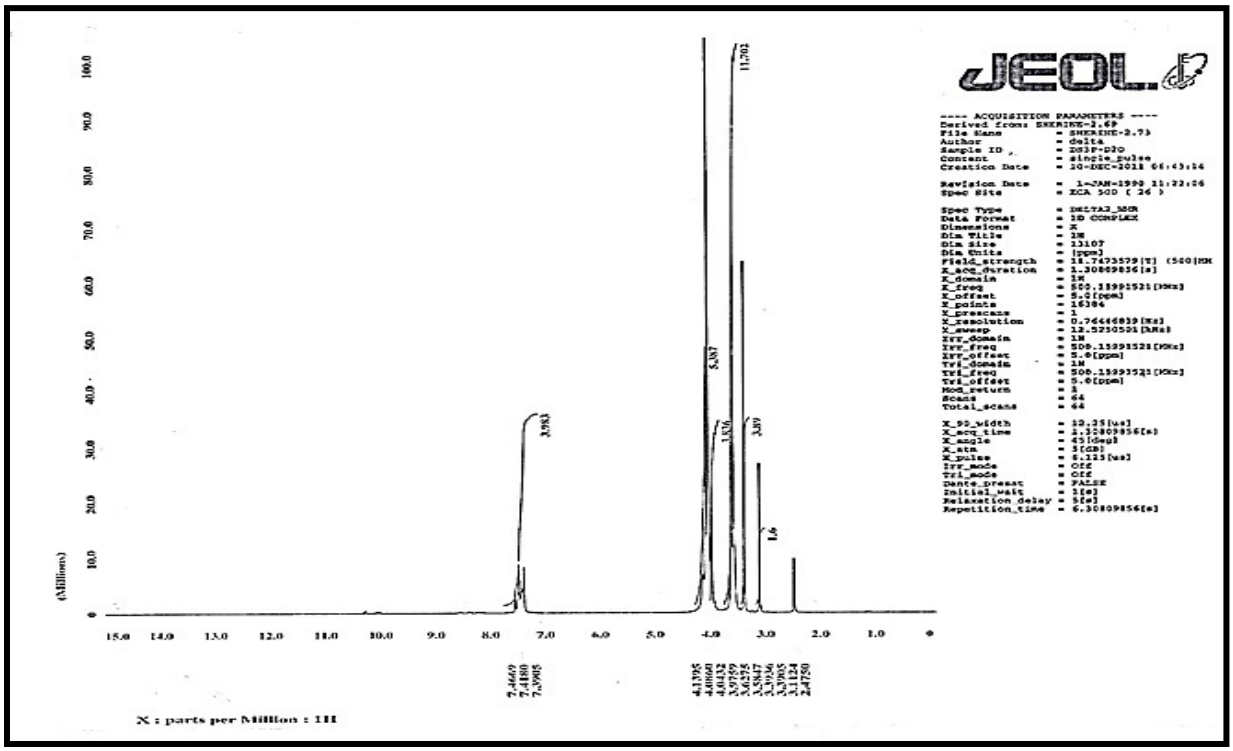




S4- IR (KBr), ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) of (TPhyG$_1$-OMe) **7**



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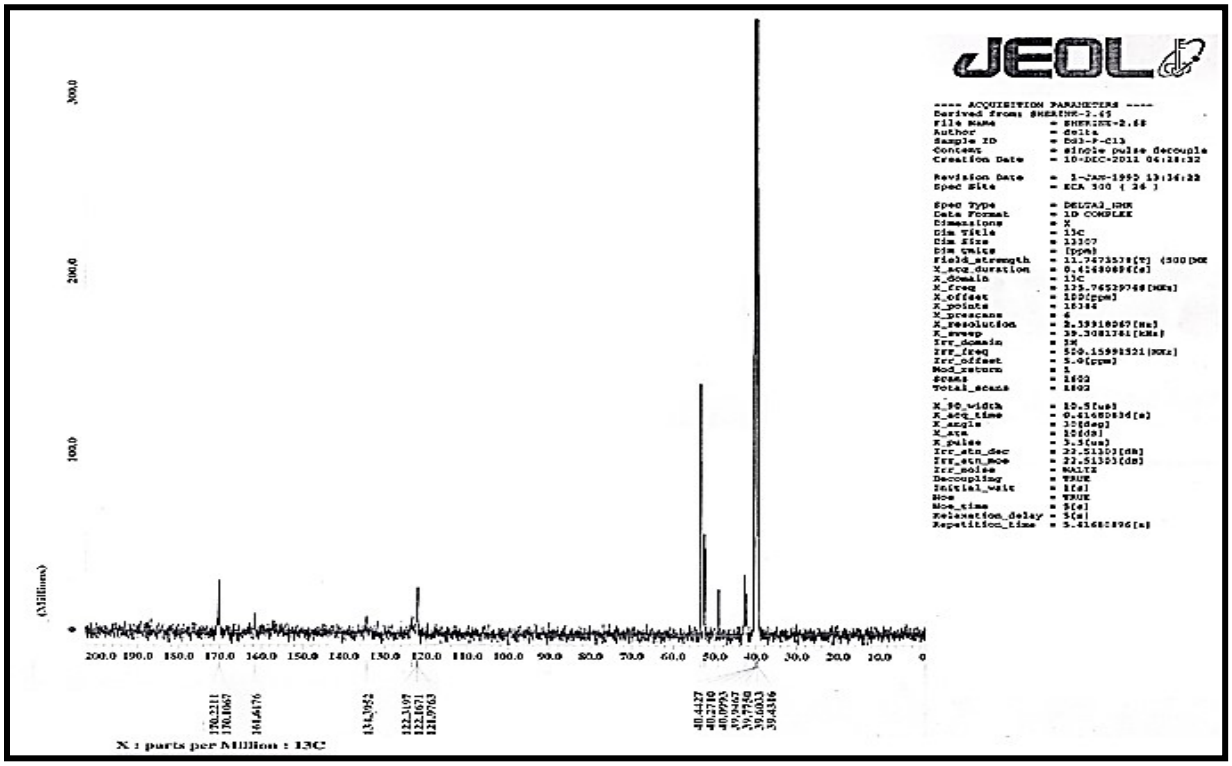
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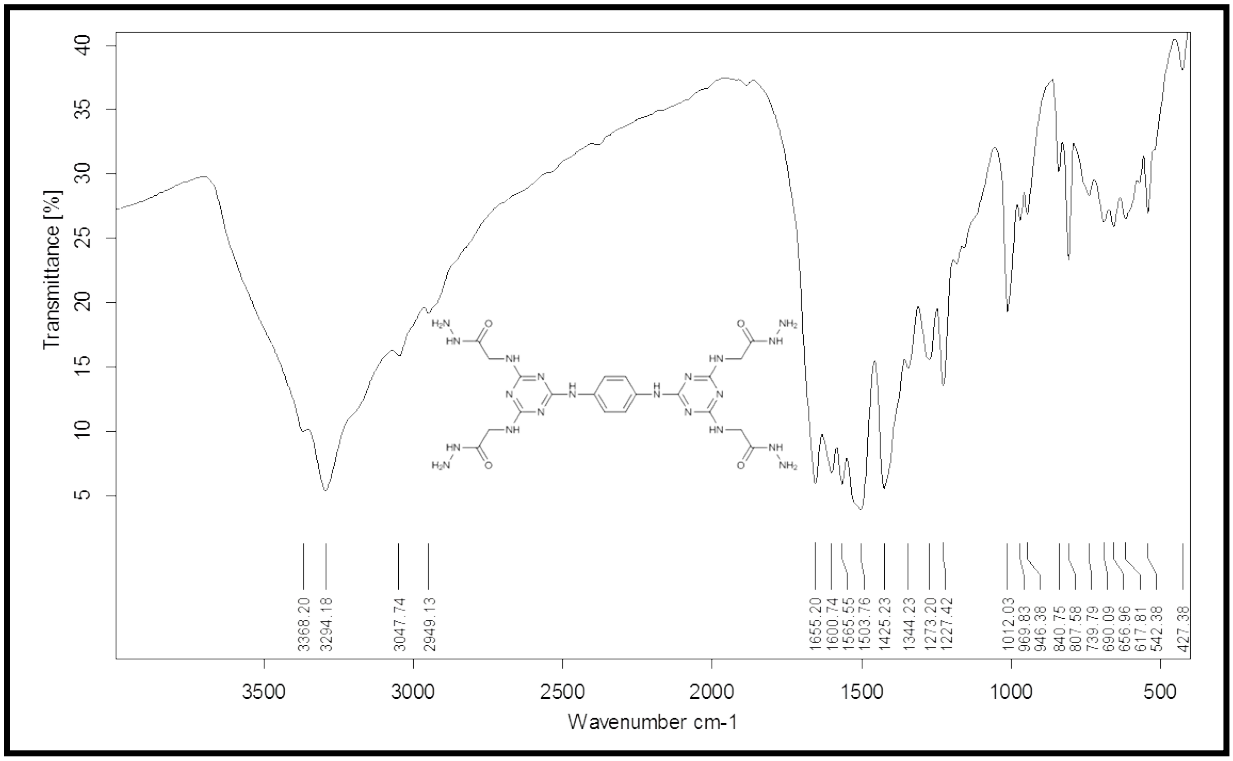
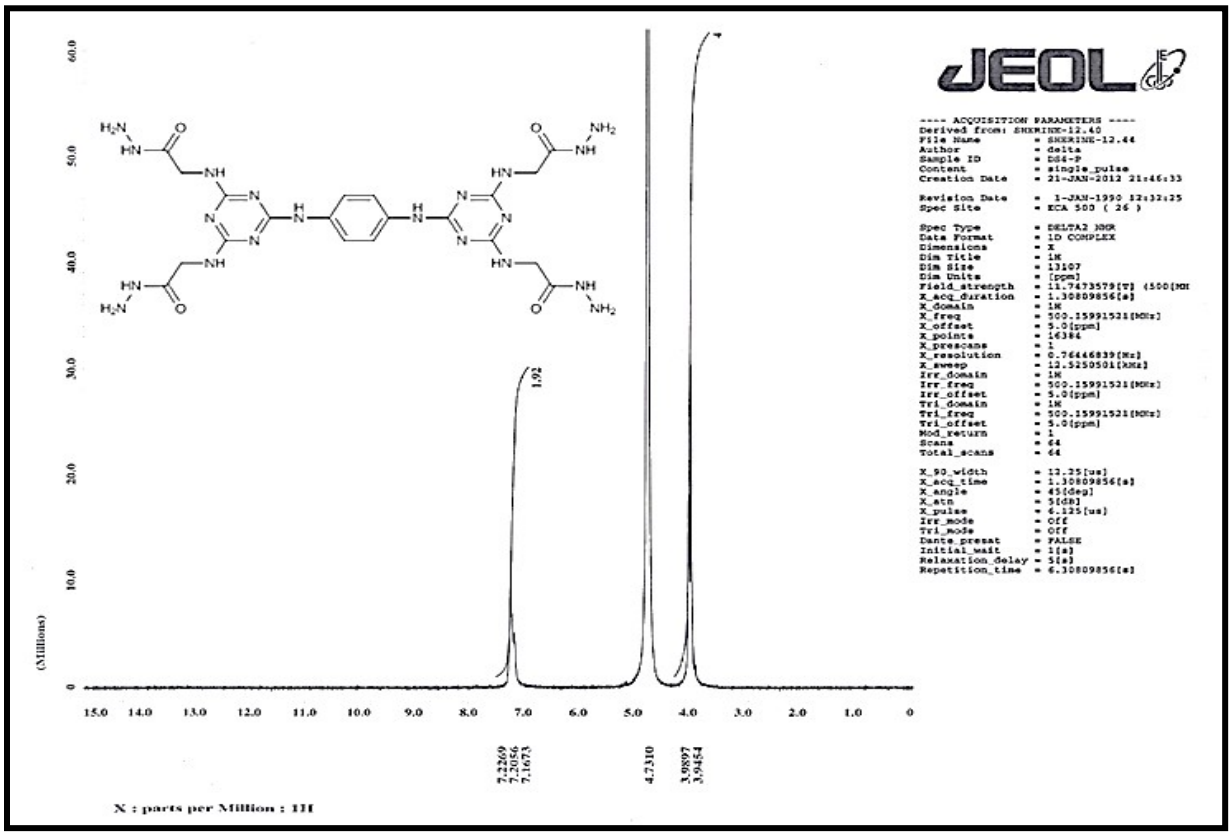
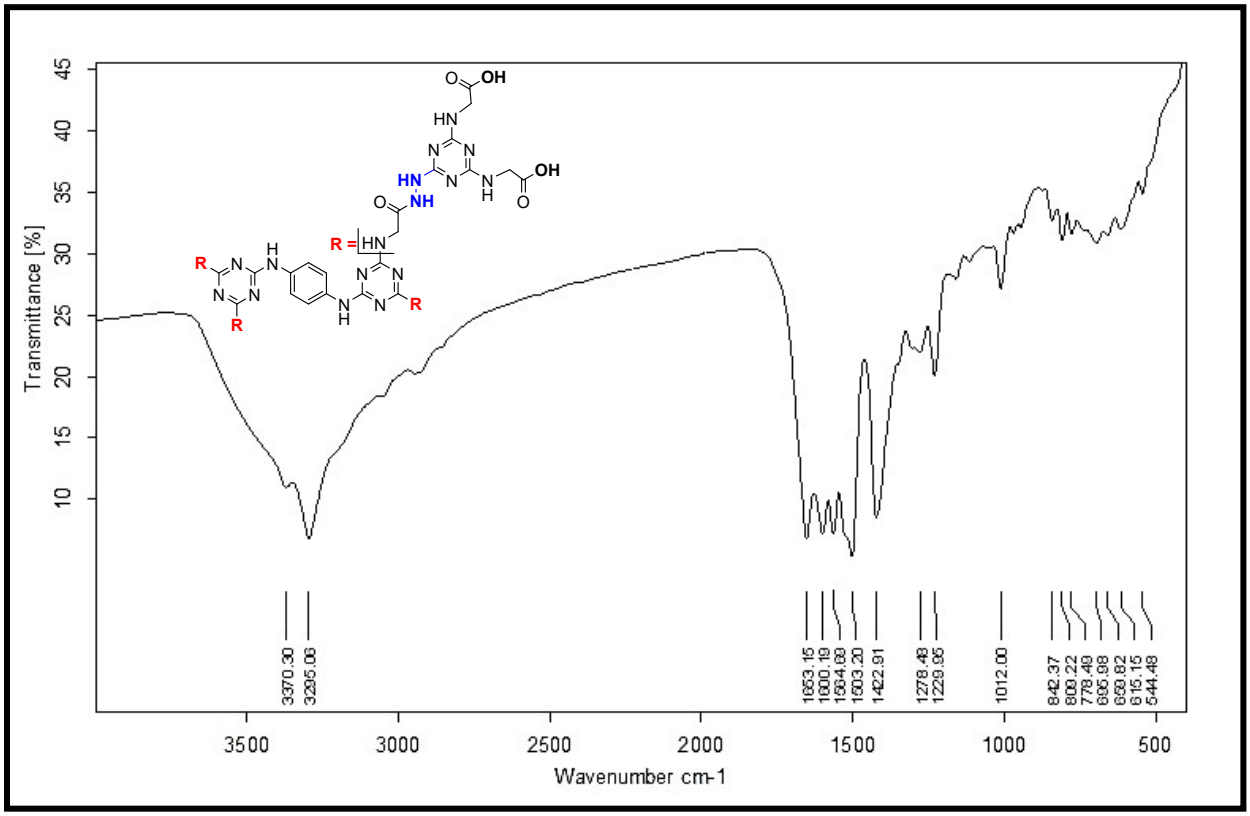
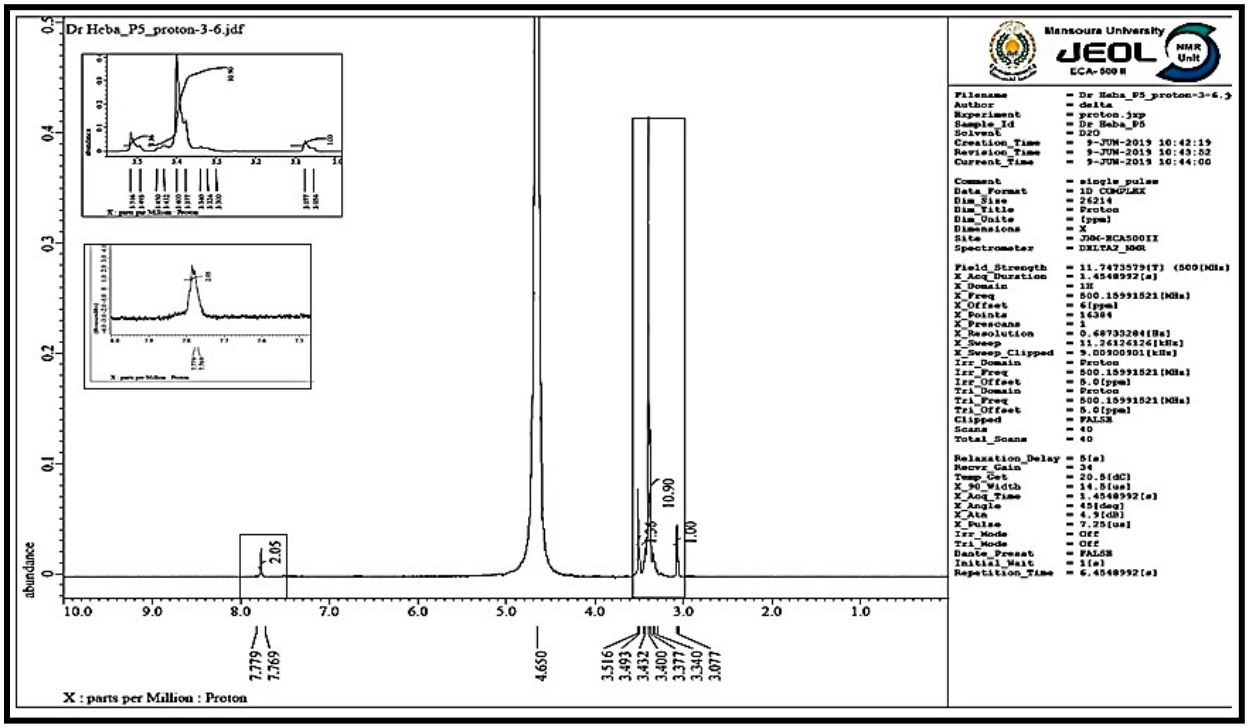
S5- IR (KBr) and ^{1}H-NMR (D_2O) of ($TPhyG_1$-NH_2) **8**

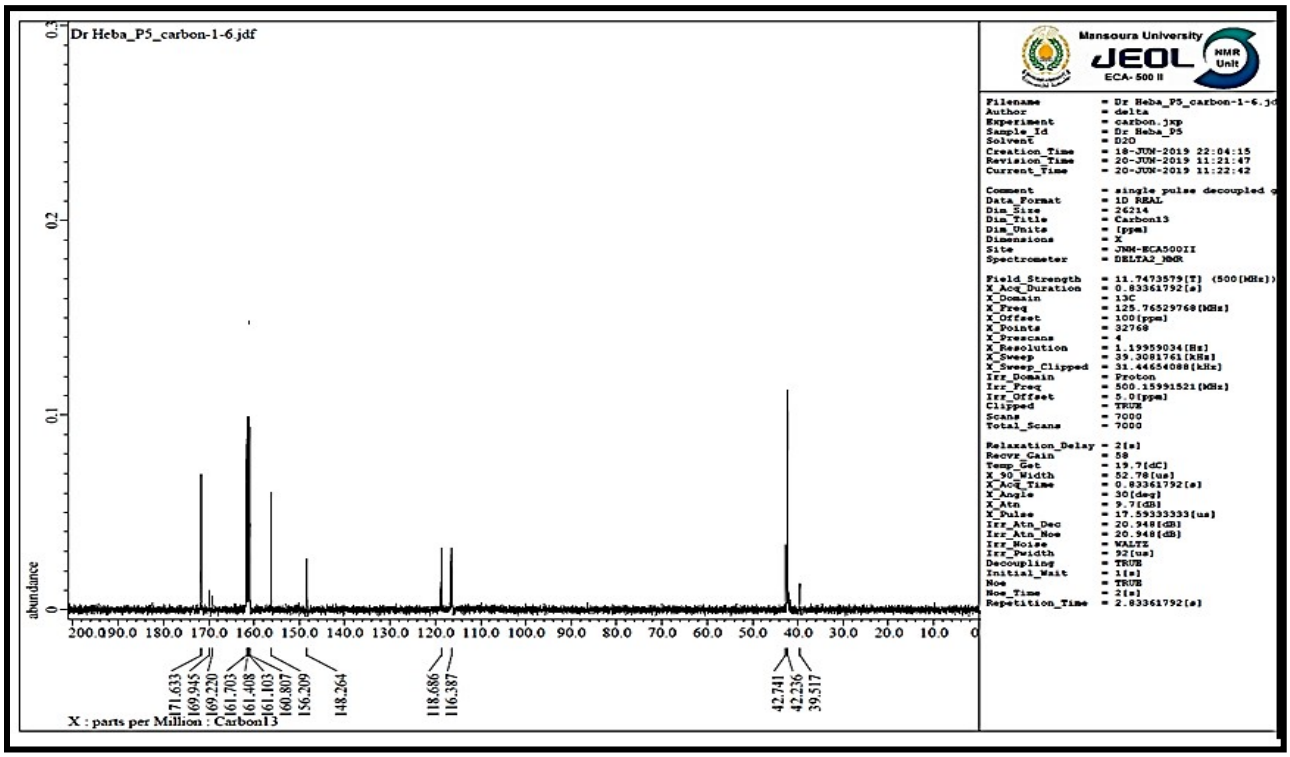




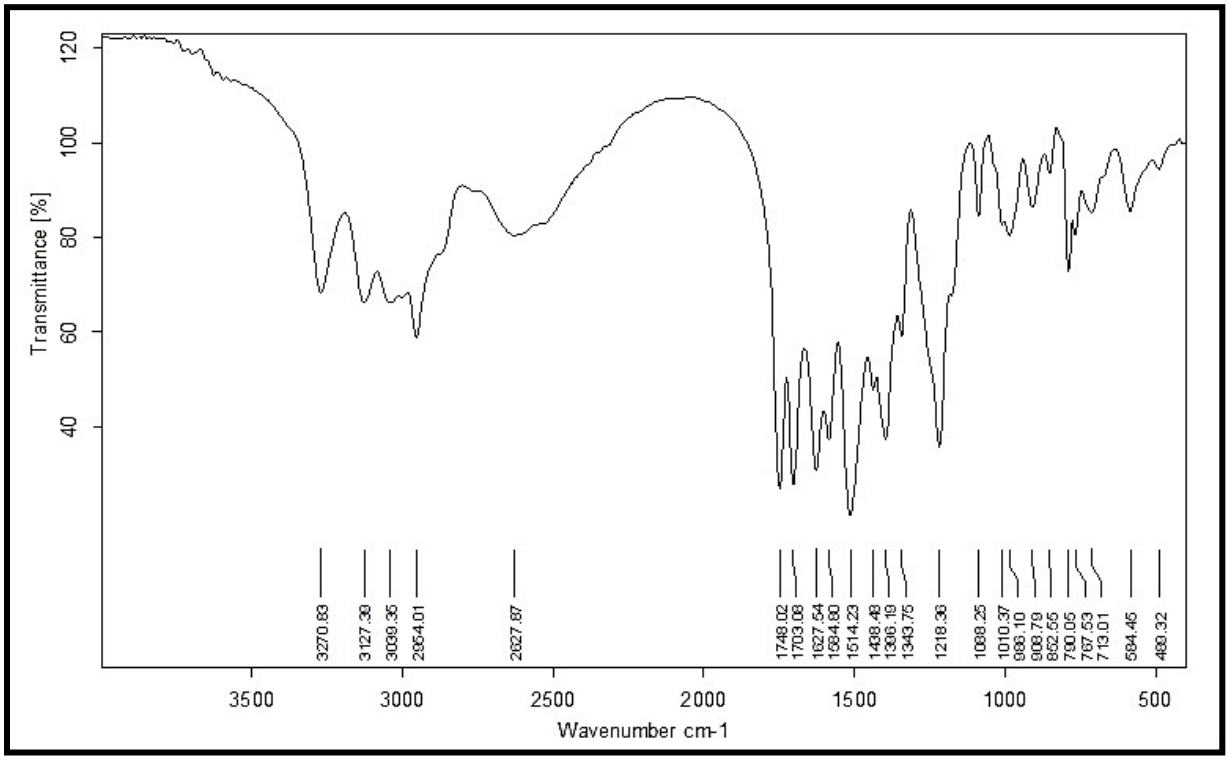
S6- IR (KBr), ^{1}H-NMR (D_2O) and ^{13}C-NMR (D_2O) of (TPhyG$_2$-OH) **9**

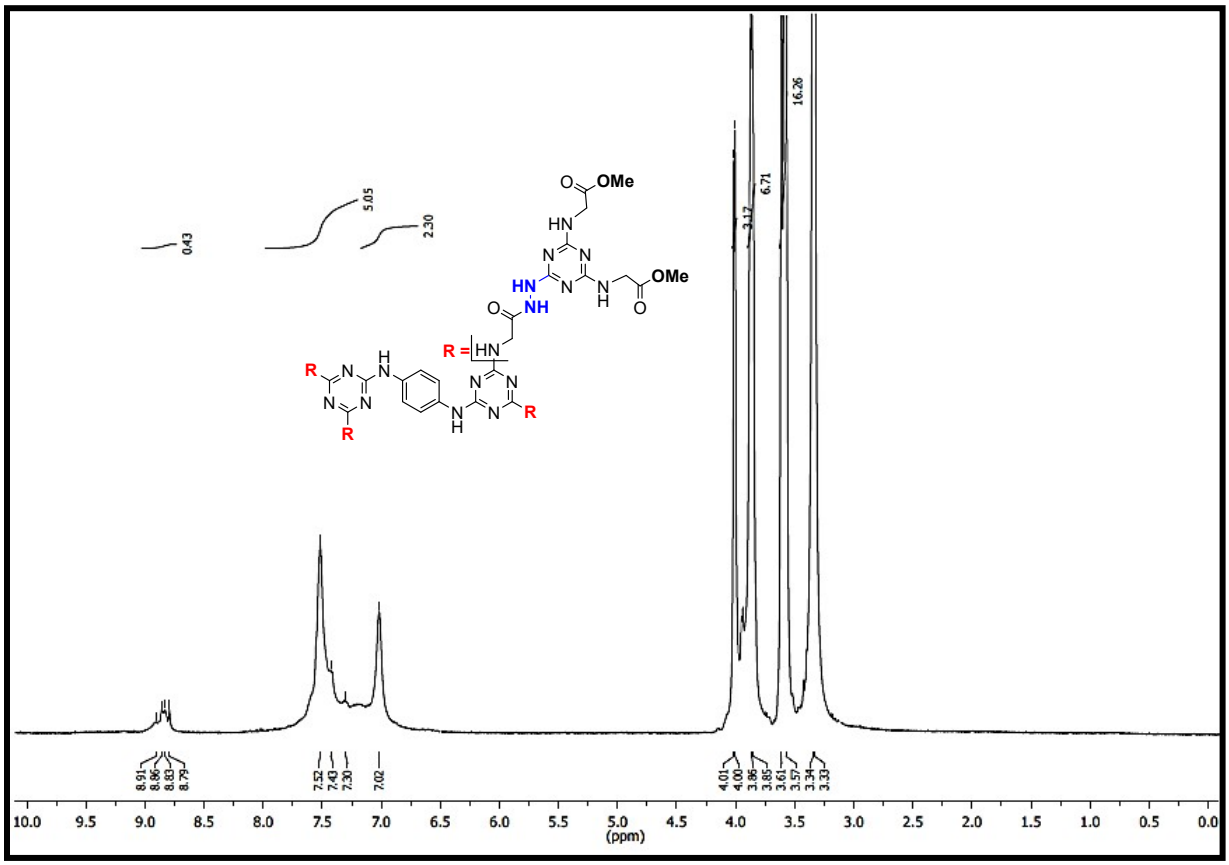


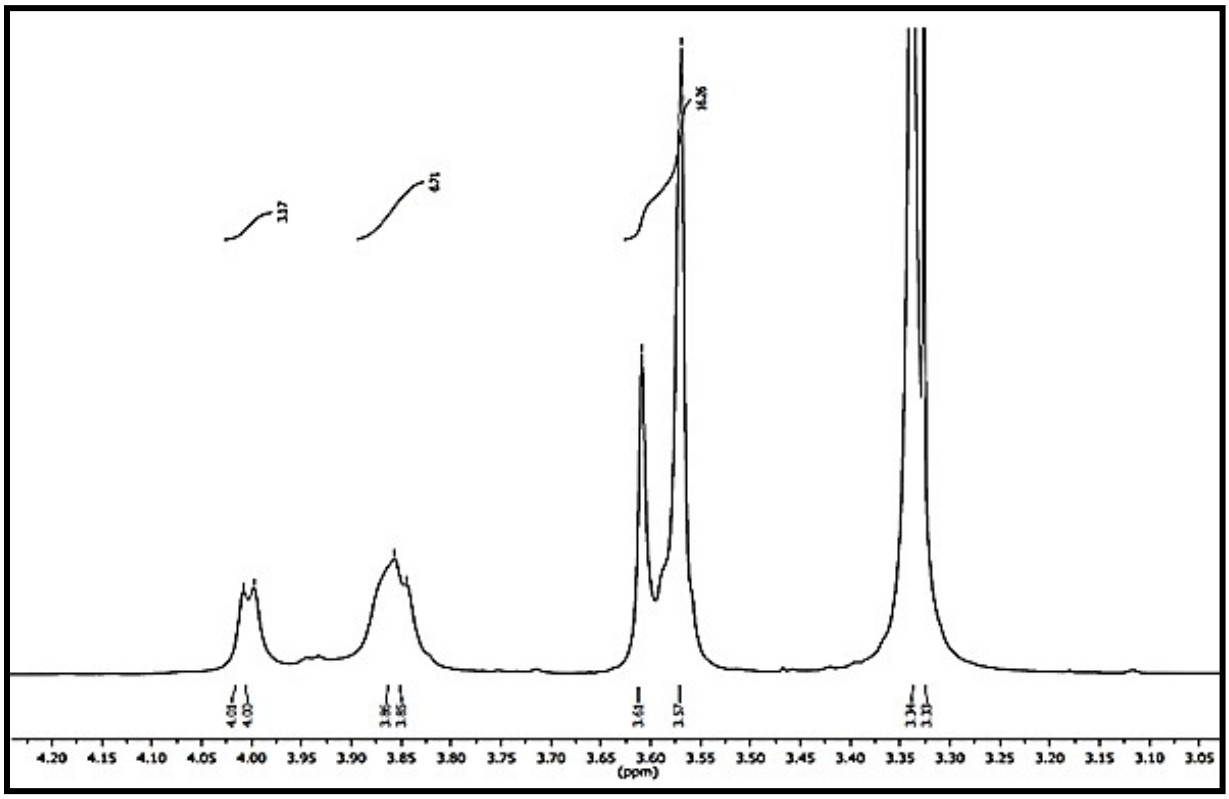


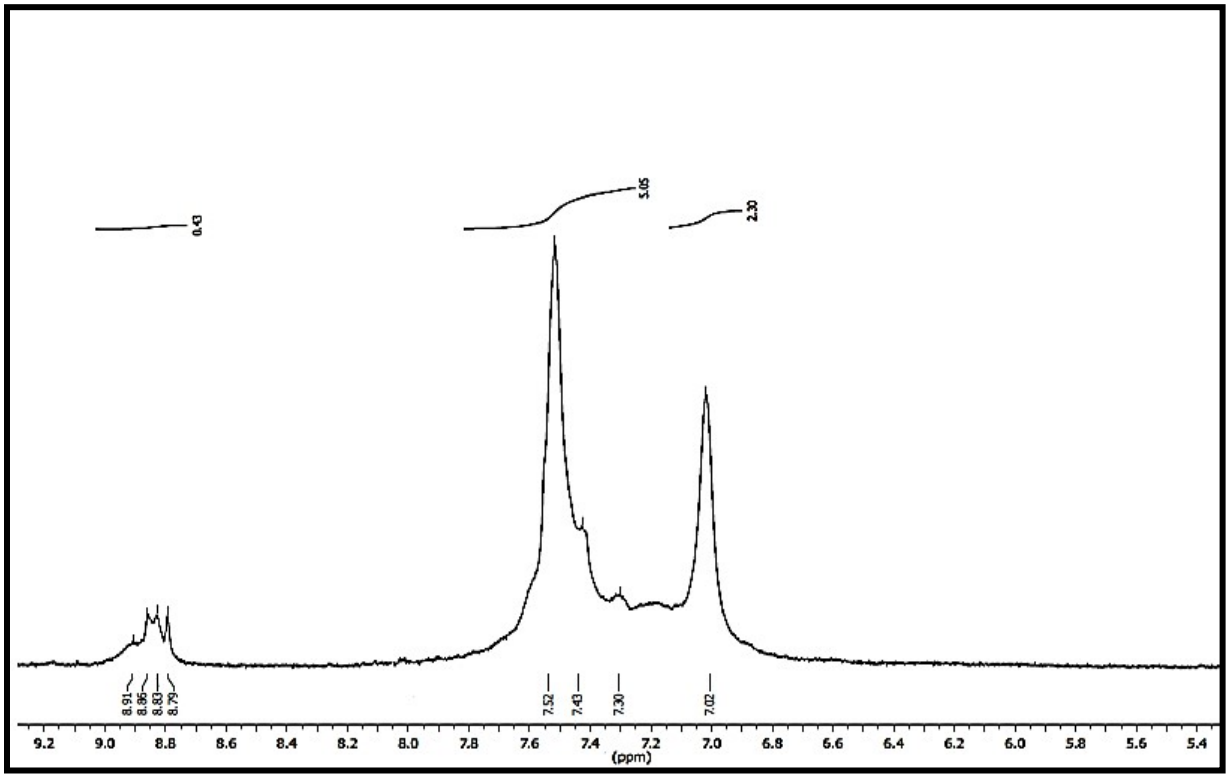

Dr Heba_P5_carbon-1-6.jdf
abundance
0.3
0.2
0.1
0
200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0 0
171.633
169.945
169.220
161.703
161.408
161.103
160.807
156.209
148.264
118.686
116.387
42.741
42.236
39.517
X : parts per Million : Carbon13
Mansoura University
JEOL
ECA- 500 II
NMR Unit
Filename = Dr Heba_P5_carbon-1-6.jd
Author = delta
Experiment = carbon.jxp
Sample_Id = Dr Heba_P5
Solvent = D2O
Creation_Time = 18-JUN-2019 22:04:15
Revision_Time = 20-JUN-2019 11:21:47
Current_Time = 20-JUN-2019 11:22:42
Comment = single pulse decoupled g
Data_Format = 1D REAL
Dim_Size = 26214
Dim_Title = Carbon13
Dim_Units = [ppm]
Dimensions = X
Site = JNM-ECA500II
Spectrometer = DELTA2_NMR
Field_Strength = 11.7473575[T] (500[MHz])
X_Acq_Duration = 0.83361792[s]
X_Domain = 13C
X_Freq = 125.76529768[MHz]
X_Offset = 100[ppm]
X_Points = 32768
X_Prescans = 4
X_Resolution = 1.19959034[Hz]
X_Sweep = 39.3081761[kHz]
X_Sweep_Clipped = 31.44654088[kHz]
Irr_Domain = Proton
Irr_Freq = 500.15991521[MHz]
Irr_Offset = 5.0[ppm]
Clipped = TRUE
Scans = 7000
Total_Scans = 7000
Relaxation_Delay = 2[s]
Recvr_Gain = 58
Temp_Get = 19.7[dC]
X_90_Width = 52.78[us]
X_Acq_Time = 0.83361792[s]
X_Angle = 30[deg]
X_Atn = 9.7[dB]
X_Pulse = 17.59333333[us]
Irr_Atn_Dec = 20.948[dB]
Irr_Atn_Noe = 20.948[dB]
Irr_Noise = WALTZ
Irr_Pwidth = 92[us]
Decoupling = TRUE
Initial_Wait = 1[s]
Noe = TRUE
Noe_Time = 2[s]
Repetition_Time = 2.83361792[s]

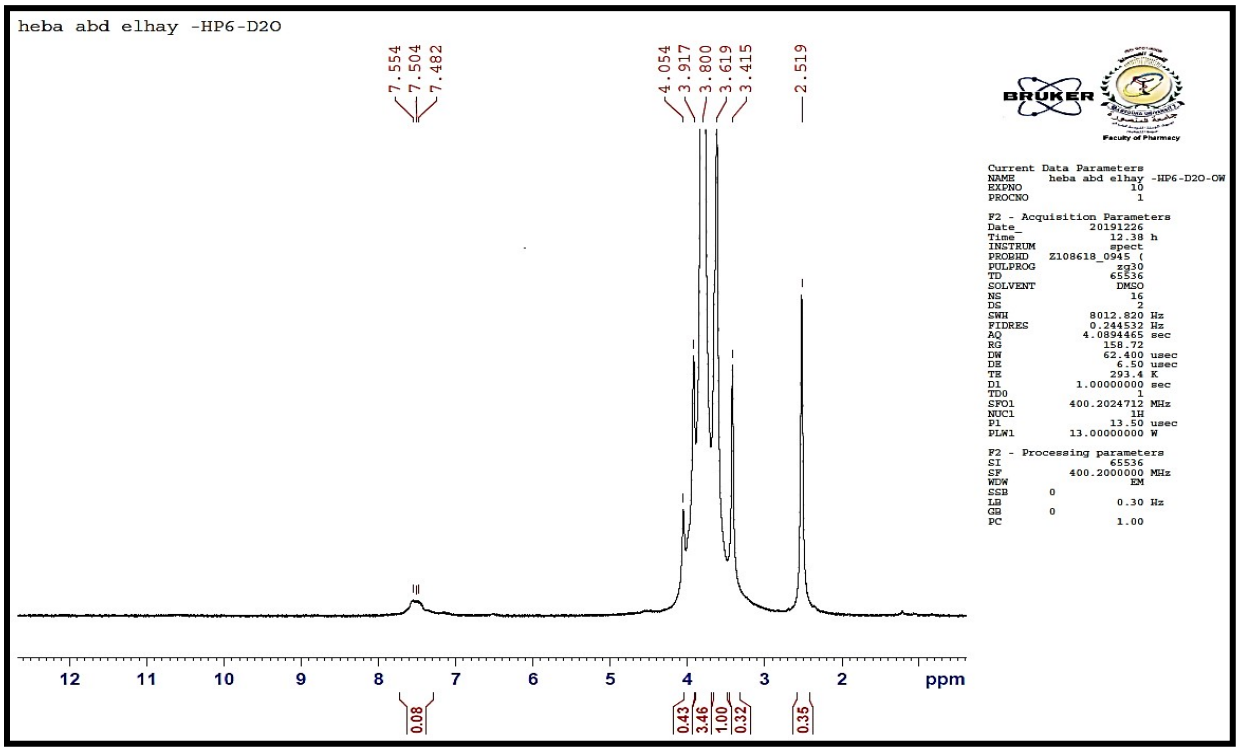
S7- IR (KBr), ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) of (TPhyG$_2$-OMe) **10**

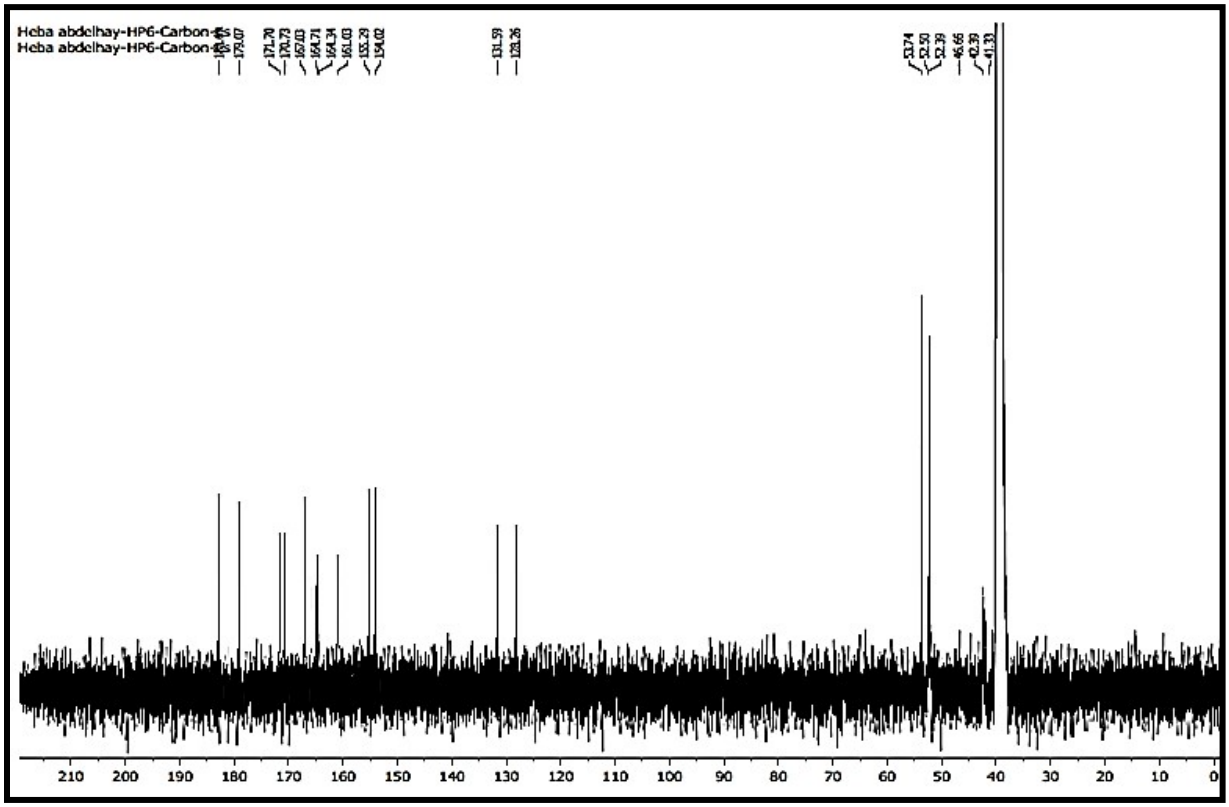




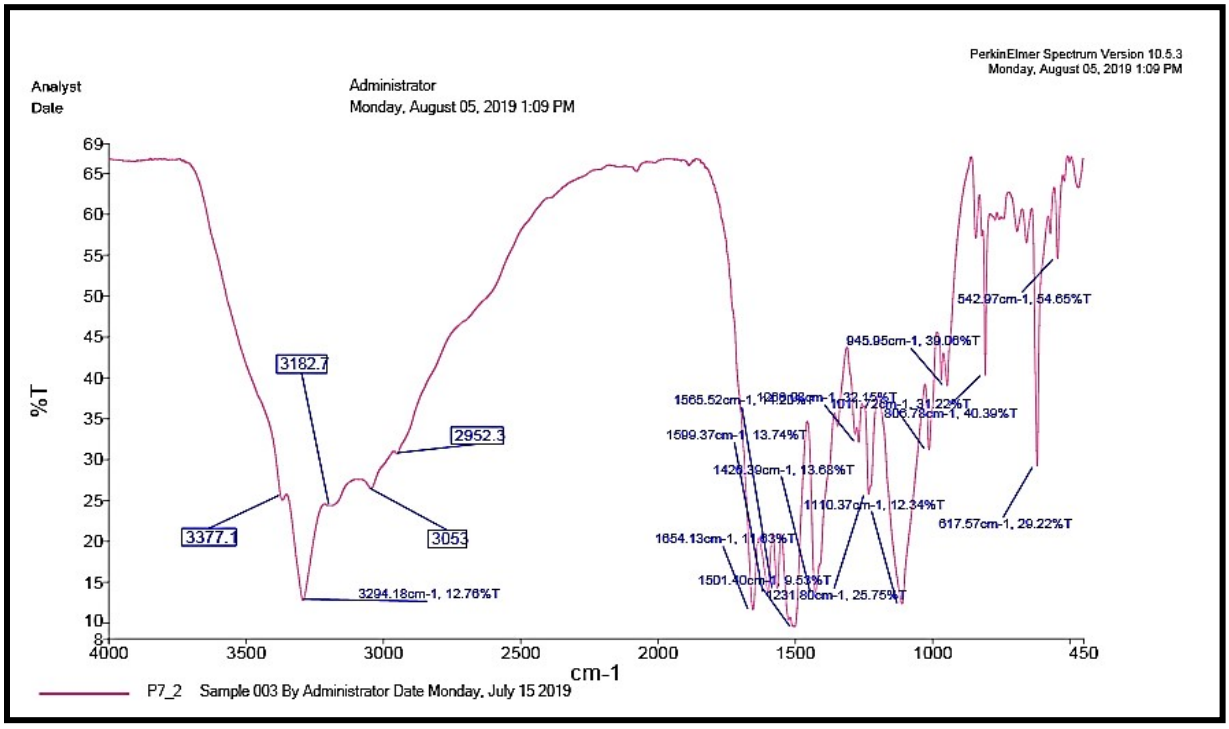

3.37
4.71
16.26
4.03
4.00
3.86
3.85
3.61
3.57
3.34
3.30
4.20 4.15 4.10 4.05 4.00 3.95 3.90 3.85 3.80 3.75 3.70 3.65 3.60 3.55 3.50 3.45 3.40 3.35 3.30 3.25 3.20 3.15 3.10 3.05
(ppm)

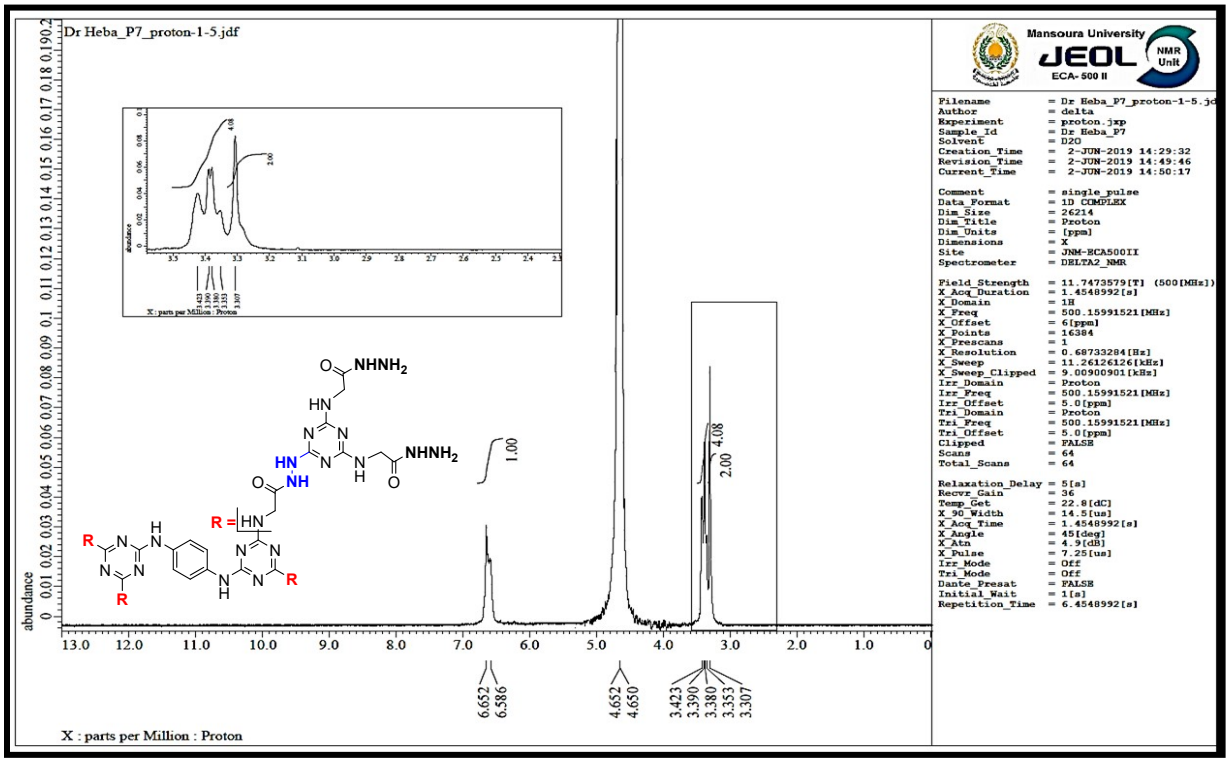

0.43
5.05
2.30
8.91
8.86
8.83
8.79
7.52
7.43
7.30
7.02
9.2 9.0 8.8 8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 5.4
(ppm)

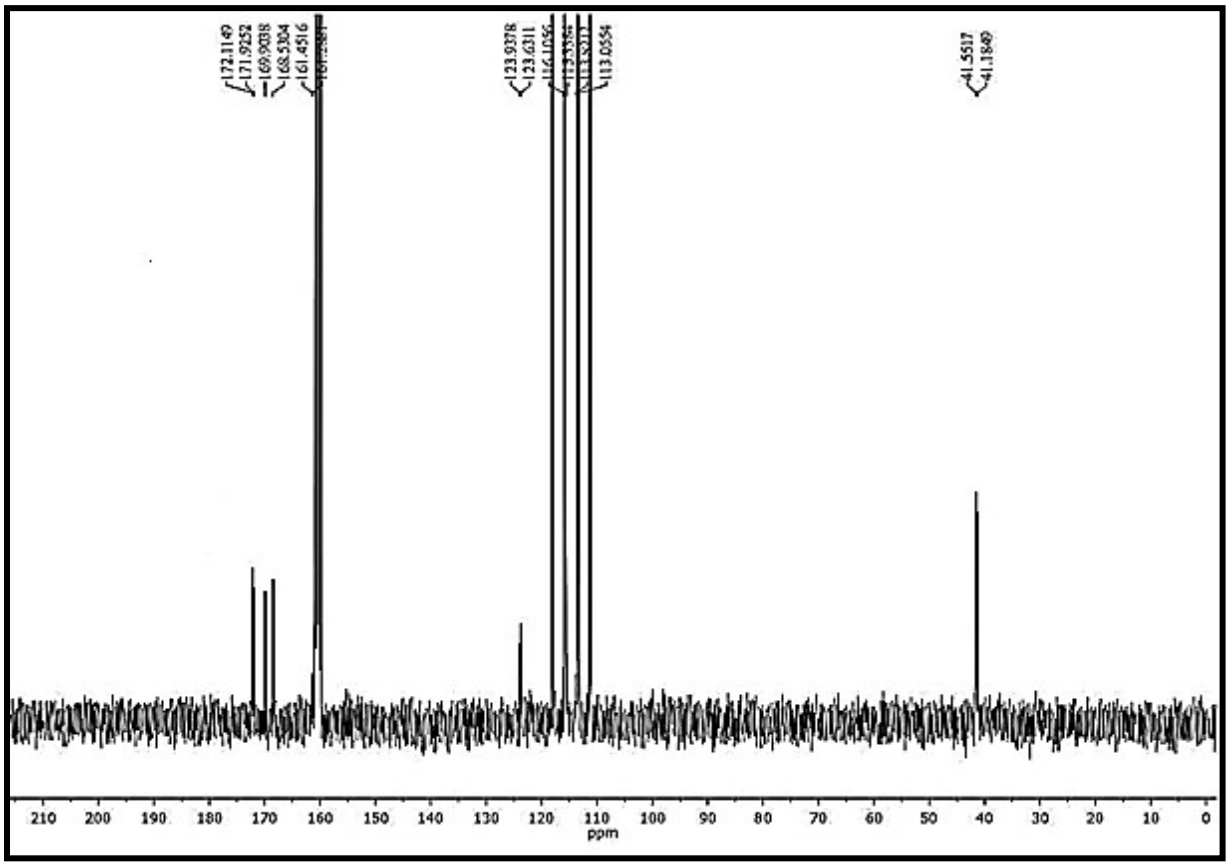

heba abd elhay -HP6-D2O
7.554
7.504
7.482
4.054
3.917
3.800
3.619
3.415
2.519
12
11
10
9
8
7
6
5
4
3
2
ppm
0.08
0.43
3.46
1.00
0.32
0.35
Current Data Parameters
NAME heba abd elhay -HP6-D2O-OW
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20191226
Time 12.38 h
INSTRUM spect
PROBHD Z108618_0945 (
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 16
DS 2
SWH 8012.820 Hz
FIDRES 0.244532 Hz
AQ 4.0894465 sec
RG 158.72
DW 62.400 usec
DE 6.50 usec
TE 293.4 K
D1 1.00000000 sec
TD0 1
SFO1 400.2024712 MHz
NUC1 1H
P1 13.50 usec
PLW1 13.00000000 W
F2 - Processing parameters
SI 65536
SF 400.2000000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00


Heba abdelhay-HP6-Carbon-ss
Heba abdelhay-HP6-Carbon-ss
173.07
171.70
170.73
167.03
164.71
164.34
161.03
155.29
154.02
131.59
123.26
53.74
52.50
52.39
46.66
42.39
41.33
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0

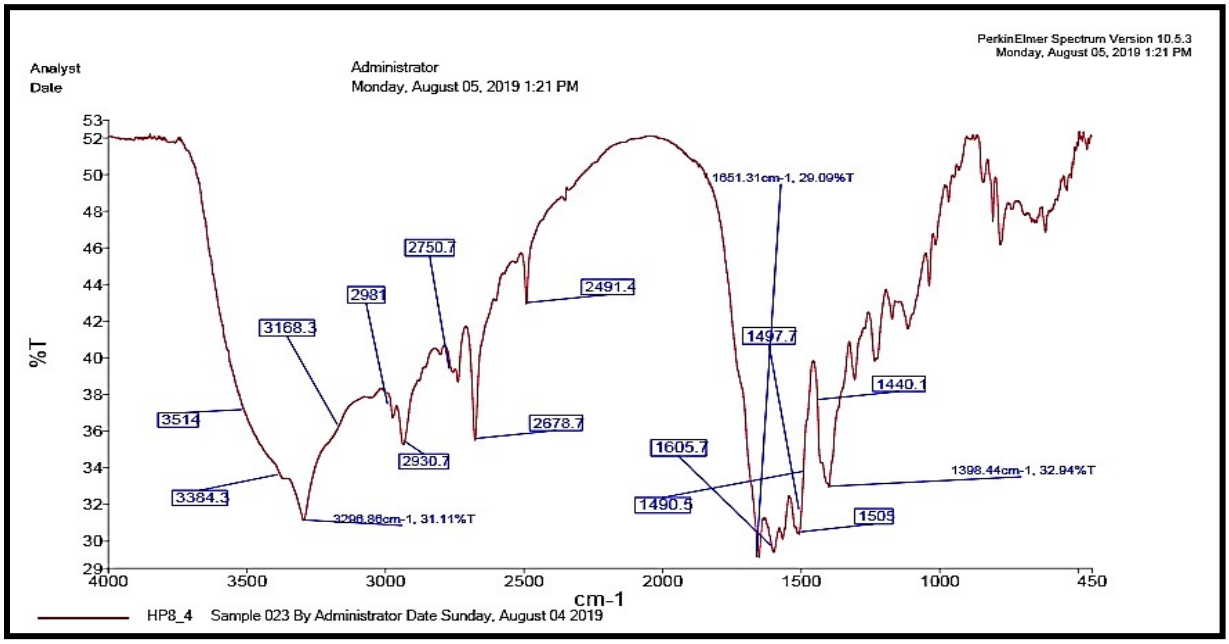
S8- IR (KBr), ^{1}H-NMR (D_2O, TFA) and ^{13}C-NMR (D_2O, TFA) of ($TPhyG_2$-NH_2) **11**

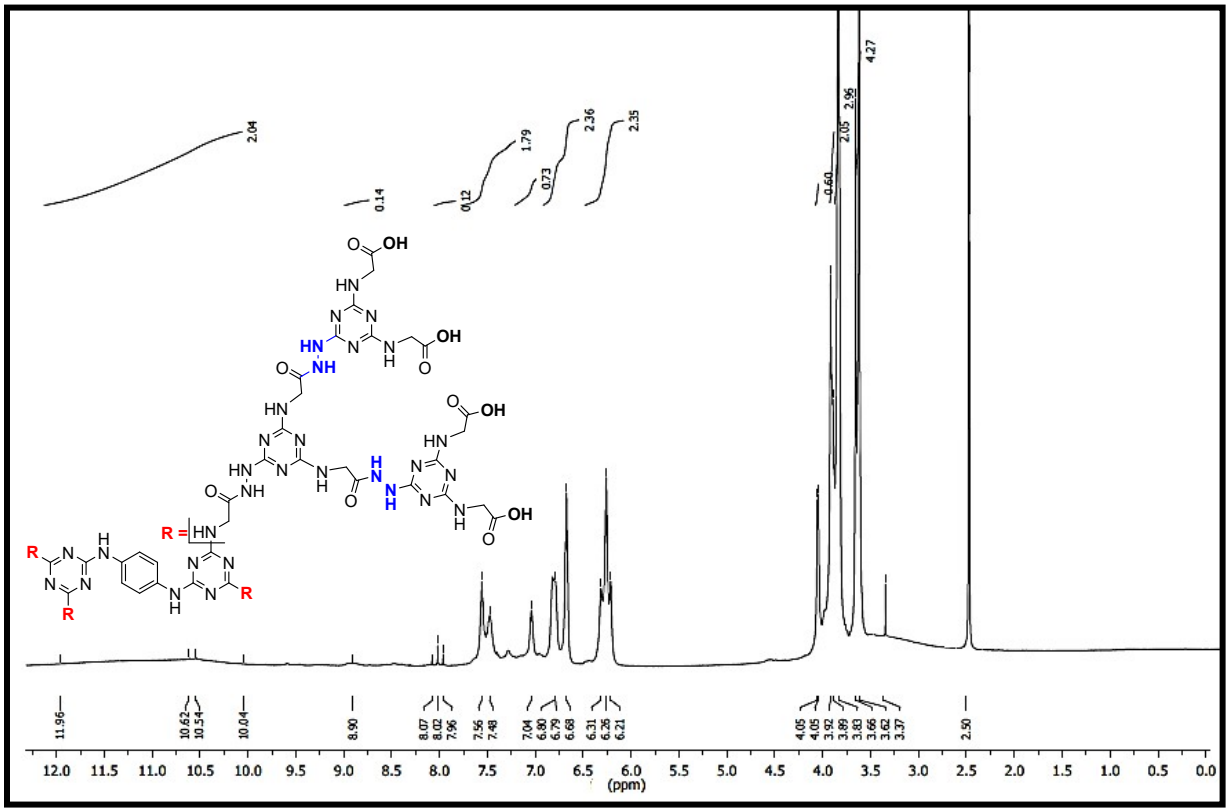


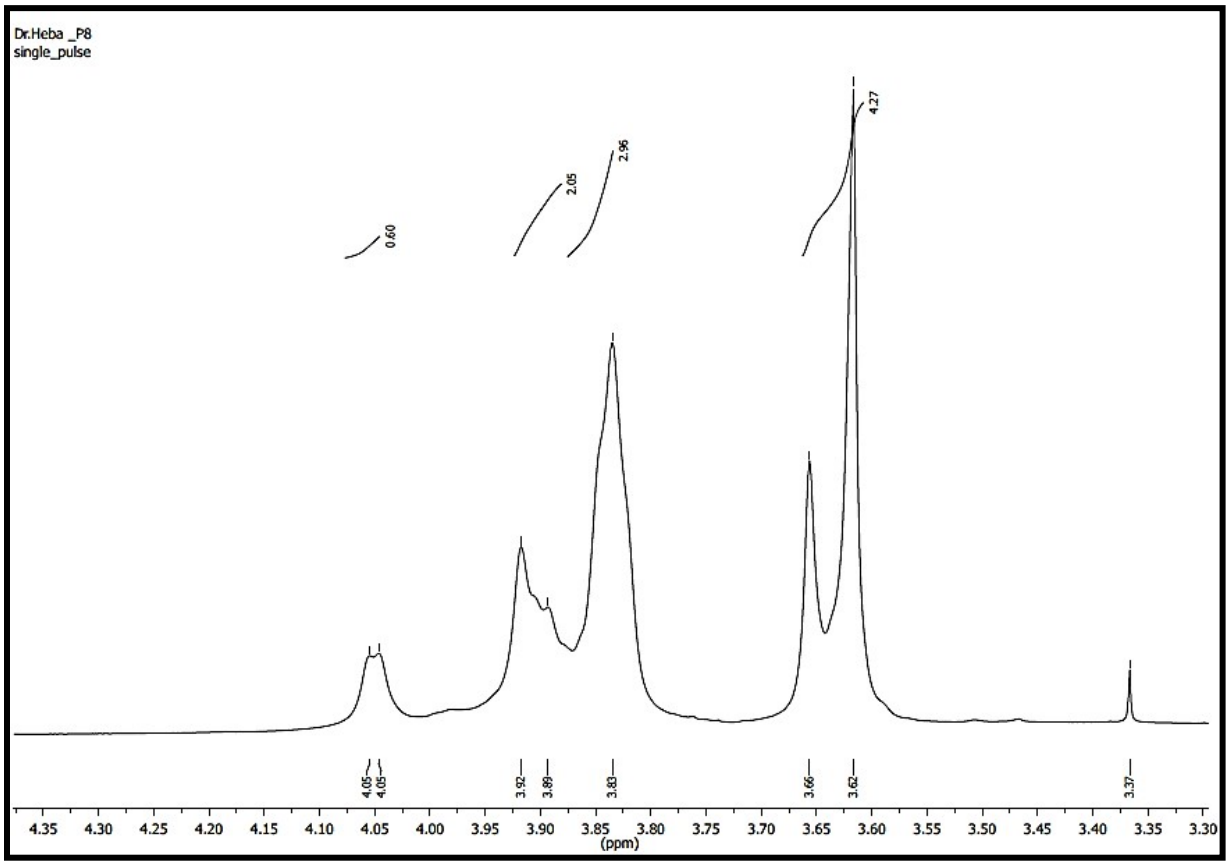


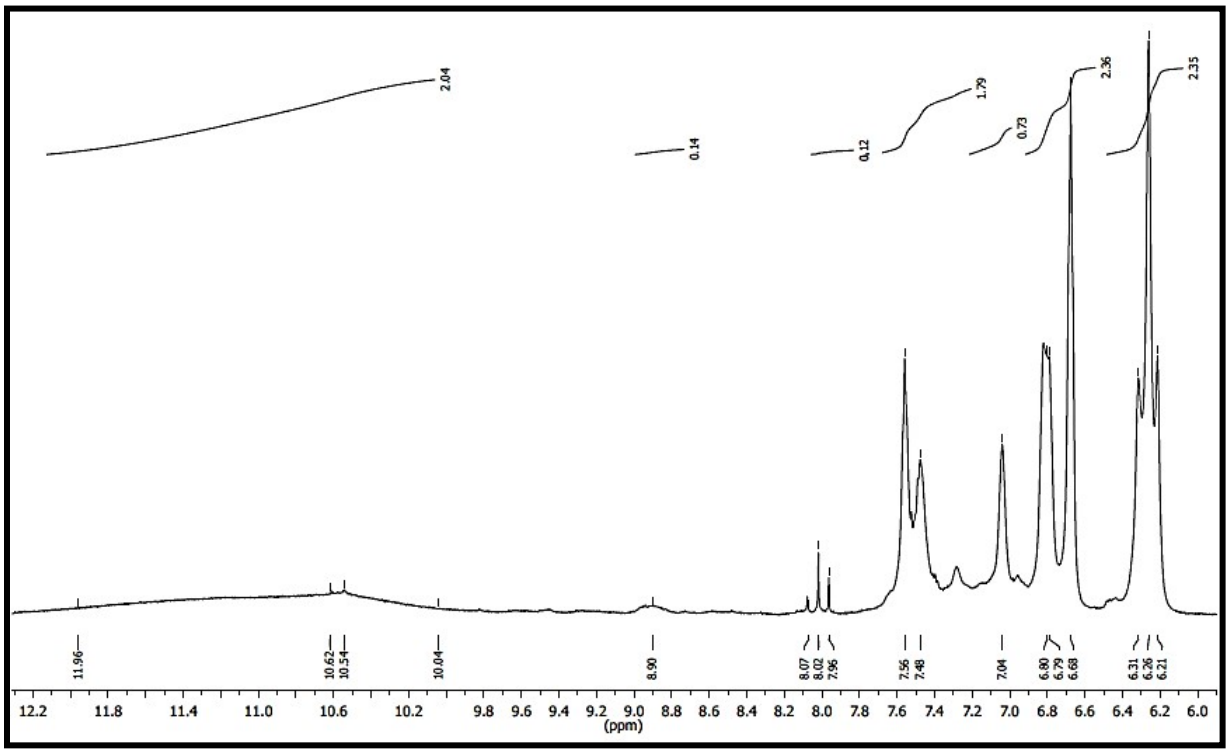

172.1149
171.9252
169.9038
168.5304
161.4516
123.9378
123.6311
113.0554
41.5517
41.1849
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm

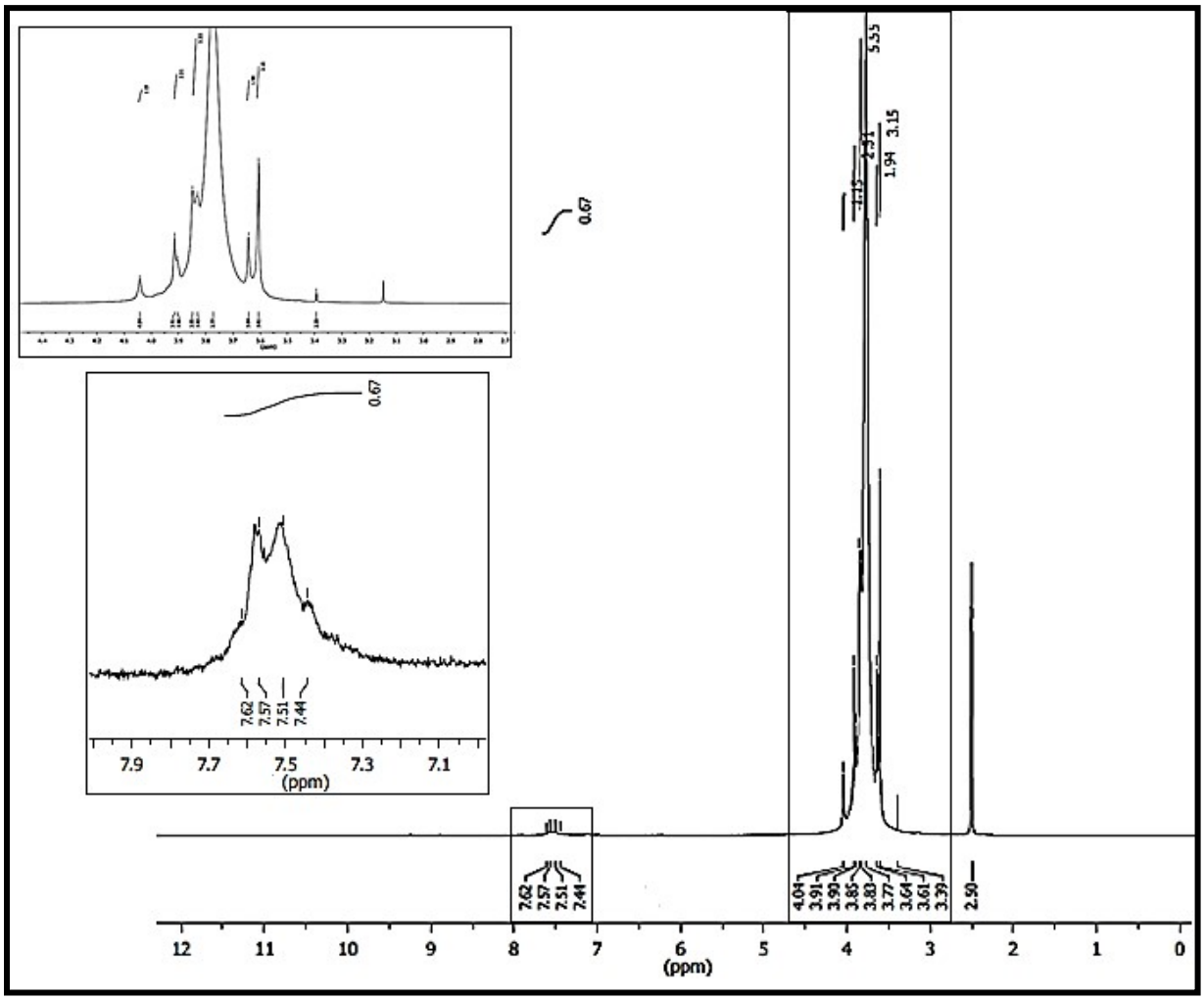
S9- IR (KBr), ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) of (TPhyG$_3$-OH) **12**

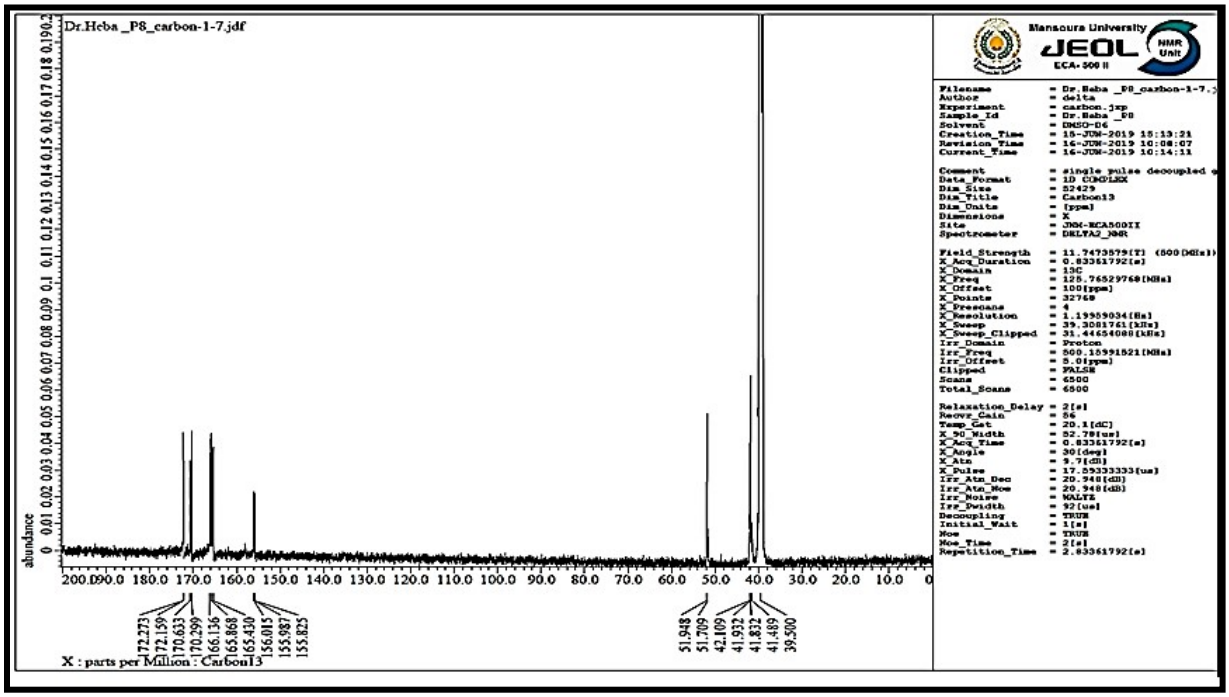




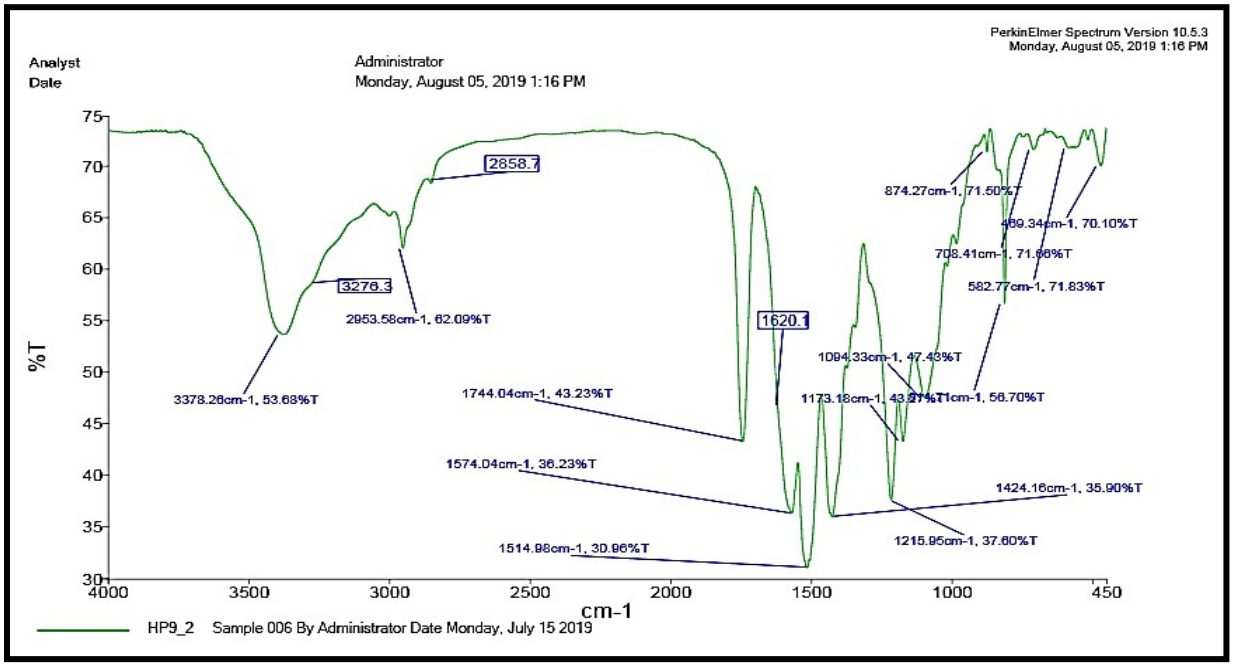

Dr.Heba _P8
single_pulse
0.60
2.05
2.96
4.27
4.05
4.05
3.92
3.89
3.83
3.66
3.62
3.37
(ppm)

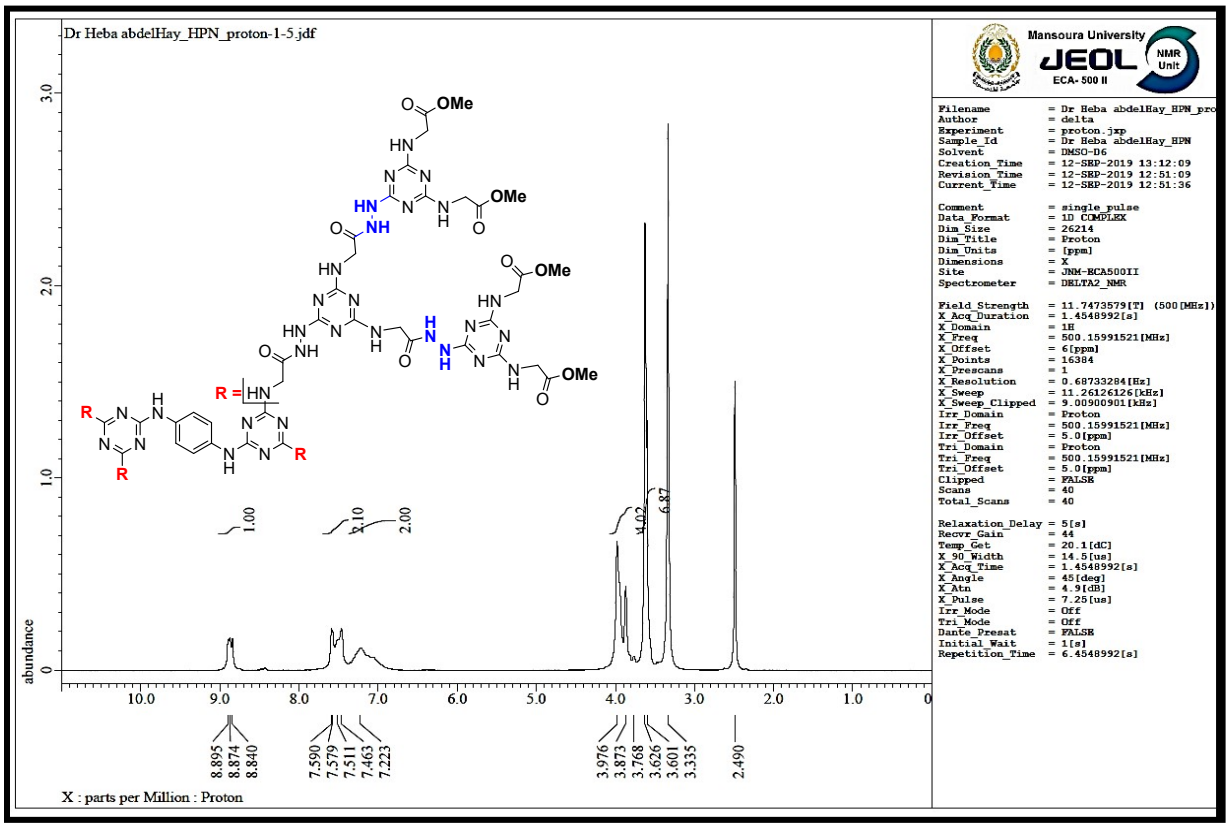

2.04
0.14
0.12
1.79
0.73
2.36
2.35
11.96
10.62
10.54
10.04
8.90
8.07
8.02
7.96
7.56
7.48
7.04
6.80
6.79
6.68
6.31
6.26
6.21
(ppm)

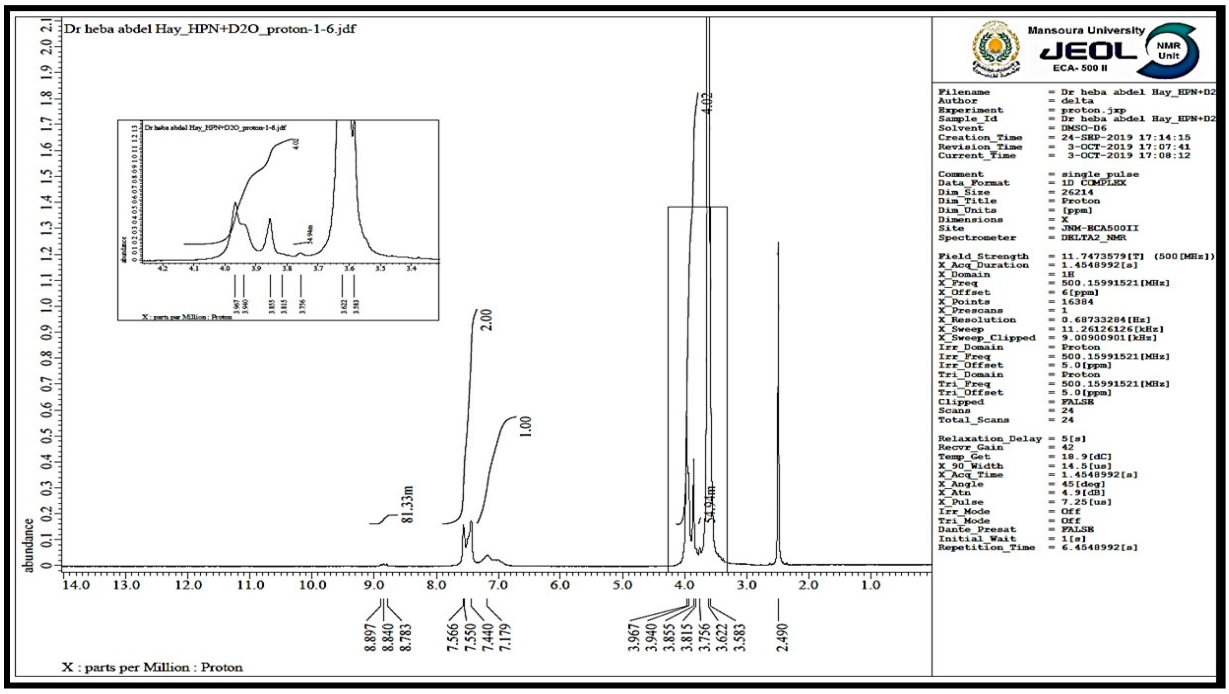

0.67
5.55
3.15
2.91
1.94
7.62
7.57
7.51
7.44
4.04
3.91
3.90
3.85
3.83
3.77
3.64
3.61
3.39
2.50
(ppm)

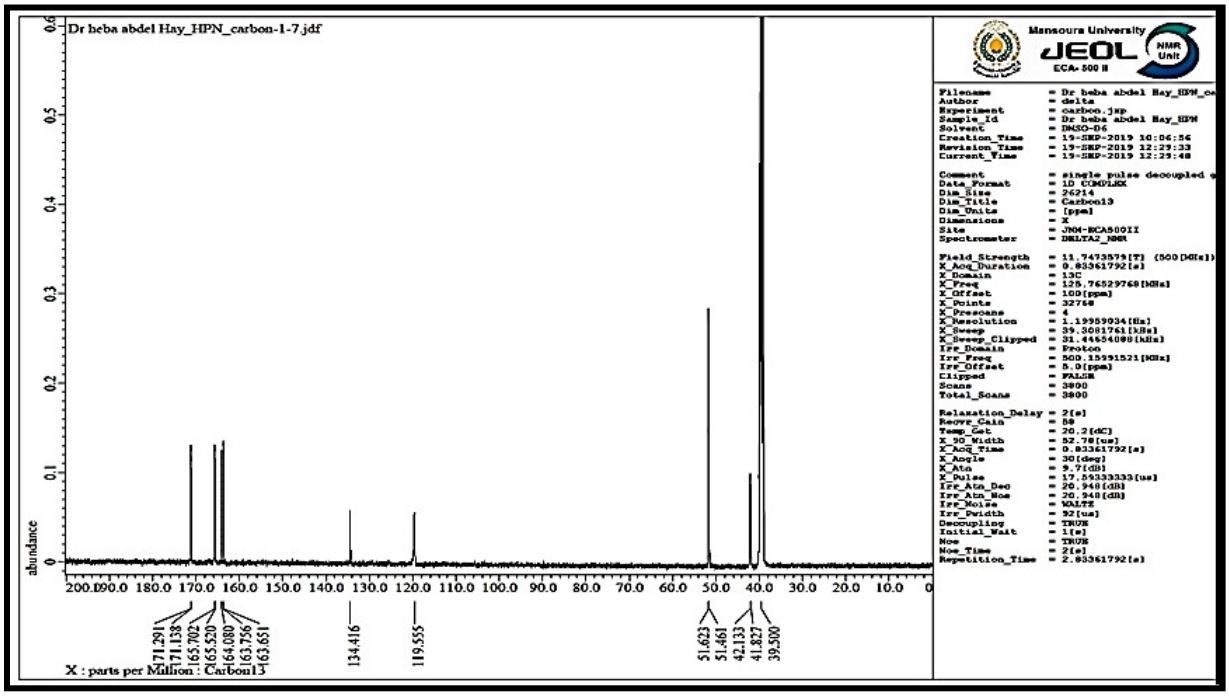

Dr.Heba _P8_carbon-1-7.jdf
Mansoura University
JEOL
ECA-500 II
NMR Unit
abundance
X : parts per Million : Carbon13
172.273
172.159
170.633
170.299
166.136
165.868
165.430
156.015
155.987
155.825
51.948
51.709
42.109
41.932
41.832
41.489
39.500

S10- IR (KBr), ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) of (TPhyG$_3$-OMe) **13**

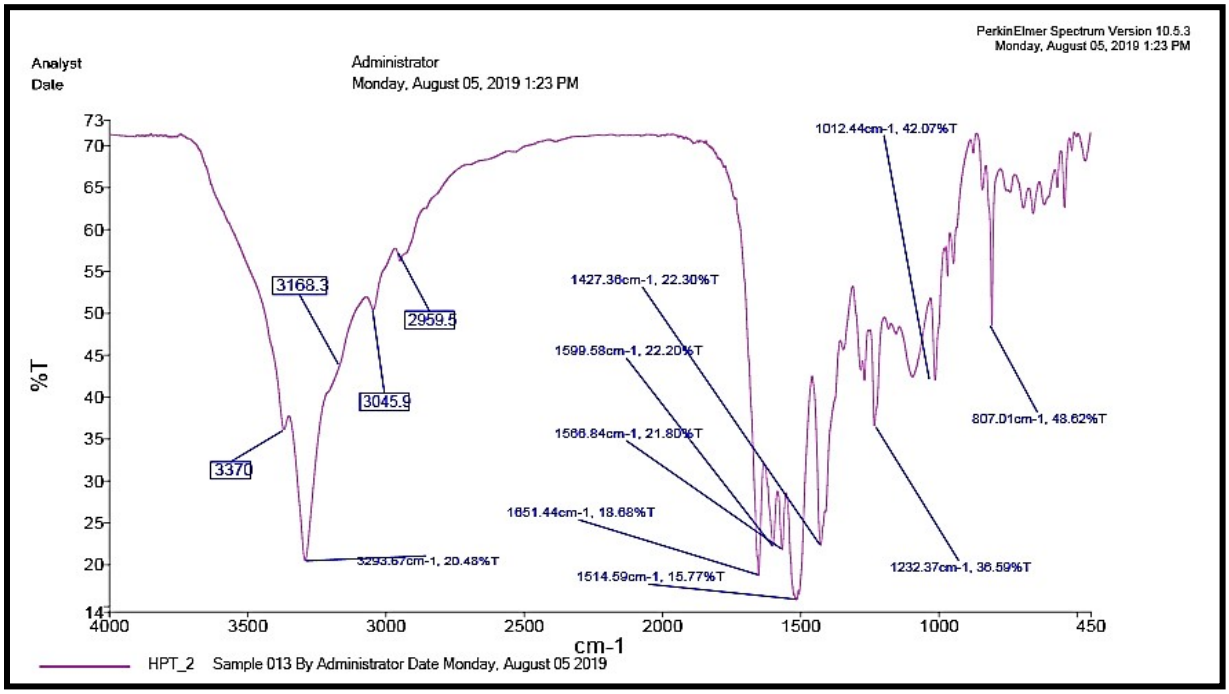


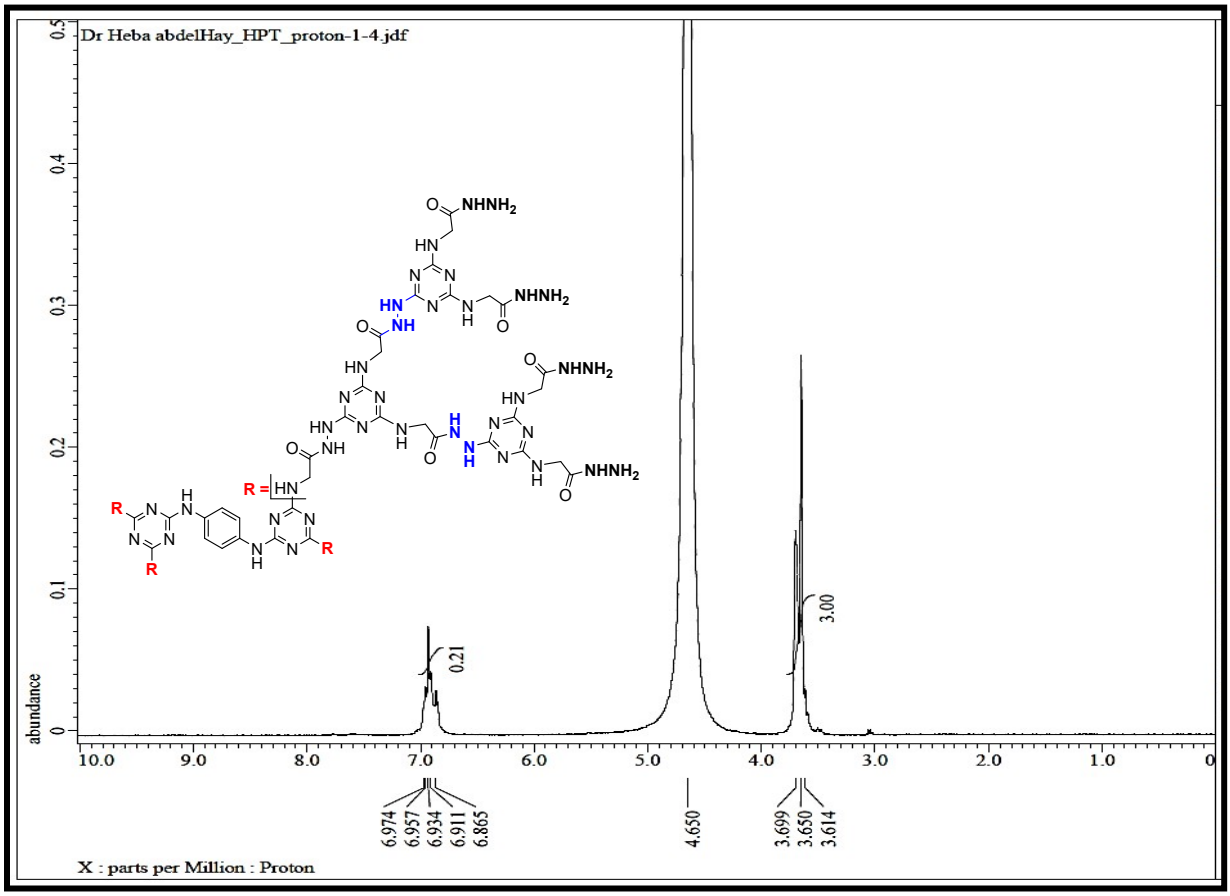


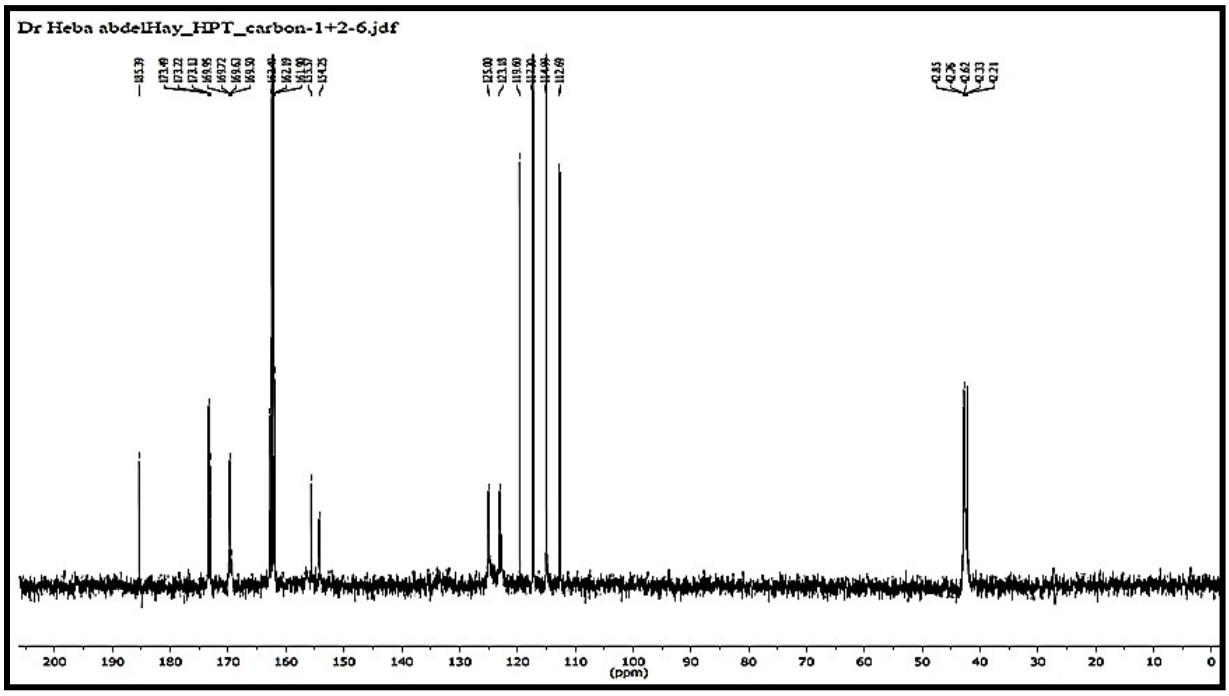

Dr heba abdel Hay_HPN+D2O_proton-1-6.jdf
Mansoura University
JEOL
NMR Unit
ECA- 500 II
Filename = Dr heba abdel Hay_HPN+D2
Author = delta
Experiment = proton.jxp
Sample_Id = Dr heba abdel Hay_HPN+D2
Solvent = DMSO-D6
Creation_Time = 24-SEP-2019 17:14:15
Revision_Time = 3-OCT-2019 17:07:41
Current_Time = 3-OCT-2019 17:08:12
Comment = single pulse
Data_Format = 1D COMPLEX
Dim_Size = 26214
Dim_Title = Proton
Dim_Units = [ppm]
Dimensions = X
Site = JNM-ECA500II
Spectrometer = DELTA2_NMR
Field_Strength = 11.7473579[T] (500[MHz])
X_Acq_Duration = 1.4548992[s]
X_Domain = 1H
X_Freq = 500.15991521[MHz]
X_Offset = 6[ppm]
X_Points = 16384
X_Prescans = 1
X_Resolution = 0.68733284[Hz]
X_Sweep = 11.26126126[kHz]
X_Sweep_Clipped = 9.00900901[kHz]
Irr_Domain = Proton
Irr_Freq = 500.15991521[MHz]
Irr_Offset = 5.0[ppm]
Tri_Domain = Proton
Tri_Freq = 500.15991521[MHz]
Tri_Offset = 5.0[ppm]
Clipped = FALSE
Scans = 24
Total_Scans = 24
Relaxation_Delay = 5[s]
Recvr_Gain = 42
Temp_Get = 18.9[dC]
X_90_Width = 14.5[us]
X_Acq_Time = 1.4548992[s]
X_Angle = 45[deg]
X_Atn = 4.9[dB]
X_Pulse = 7.25[us]
Irr_Mode = Off
Tri_Mode = Off
Dante_Presat = FALSE
Initial_Wait = 1[s]
Repetition_Time = 6.4548992[s]
abundance
X : parts per Million : Proton
8.897
8.840
8.783
7.566
7.550
7.440
7.179
3.967
3.940
3.855
3.815
3.756
3.622
3.583
2.490
81.33m
34.94m
2.00
1.00
4.03


Dr heba abdel Hay_HPN_carbon-1-7.jdf
Mansoura University
JEOL
NMR Unit
ECA- 500 II
Filename = Dr heba abdel Hay_HPN_ca
Author = delta
Experiment = carbon.jxp
Sample_Id = Dr heba abdel Hay_HPN
Solvent = DMSO-D6
Creation_Time = 19-SEP-2019 10:06:56
Revision_Time = 19-SEP-2019 12:29:33
Current_Time = 19-SEP-2019 12:29:48
Comment = single pulse decoupled g
Data_Format = 1D COMPLEX
Dim_Size = 26214
Dim_Title = Carbon13
Dim_Units = [ppm]
Dimensions = X
Site = JNM-ECA500II
Spectrometer = DELTA2_NMR
Field_Strength = 11.7473579[T] (500[MHz])
X_Acq_Duration = 0.83361792[s]
X_Domain = 13C
X_Freq = 125.76529768[MHz]
X_Offset = 100[ppm]
X_Points = 32768
X_Prescans = 4
X_Resolution = 1.19959034[Hz]
X_Sweep = 39.3081761[kHz]
X_Sweep_Clipped = 31.44654088[kHz]
Irr_Domain = Proton
Irr_Freq = 500.15991521[MHz]
Irr_Offset = 5.0[ppm]
Clipped = FALSE
Scans = 3800
Total_Scans = 3800
Relaxation_Delay = 2[s]
Recvr_Gain = 58
Temp_Get = 20.2[dC]
X_90_Width = 52.78[us]
X_Acq_Time = 0.83361792[s]
X_Angle = 30[deg]
X_Atn = 9.7[dB]
X_Pulse = 17.58333333[us]
Irr_Atn_Dec = 20.94[dB]
Irr_Atn_Noe = 20.94[dB]
Irr_Noise = WALTZ
Irr_Pwidth = 92[us]
Decoupling = TRUE
Initial_Wait = 1[s]
Noe = TRUE
Noe_Time = 2[s]
Repetition_Time = 2.83361792[s]
abundance
X : parts per Million : Carbon13
171.291
171.138
165.702
165.520
164.080
163.756
163.651
134.416
119.555
51.623
51.461
42.133
41.827
39.500

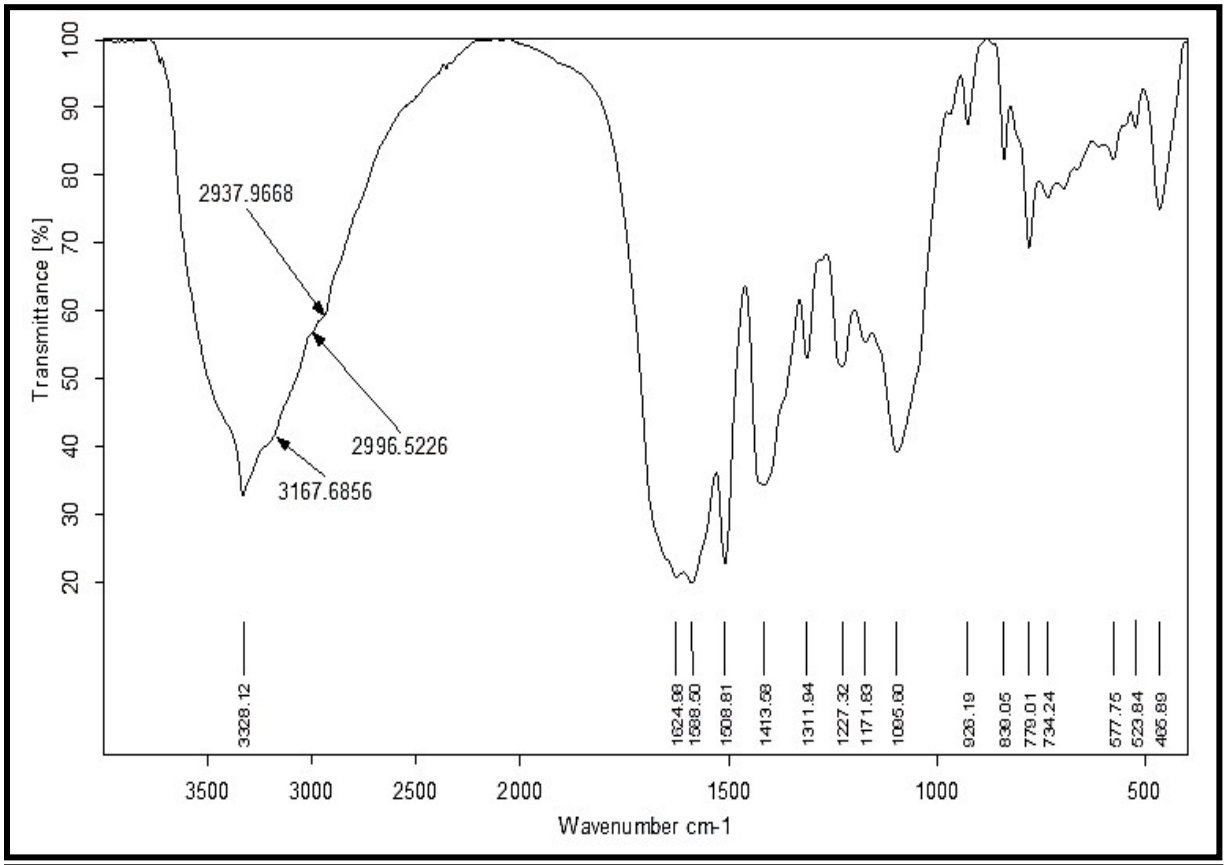
S11- IR (KBr), ^{1}H-NMR (D_2O,TFA) and ^{13}C-NMR (D_2O, TFA) of (TPhyG_3-NH_2) **14**

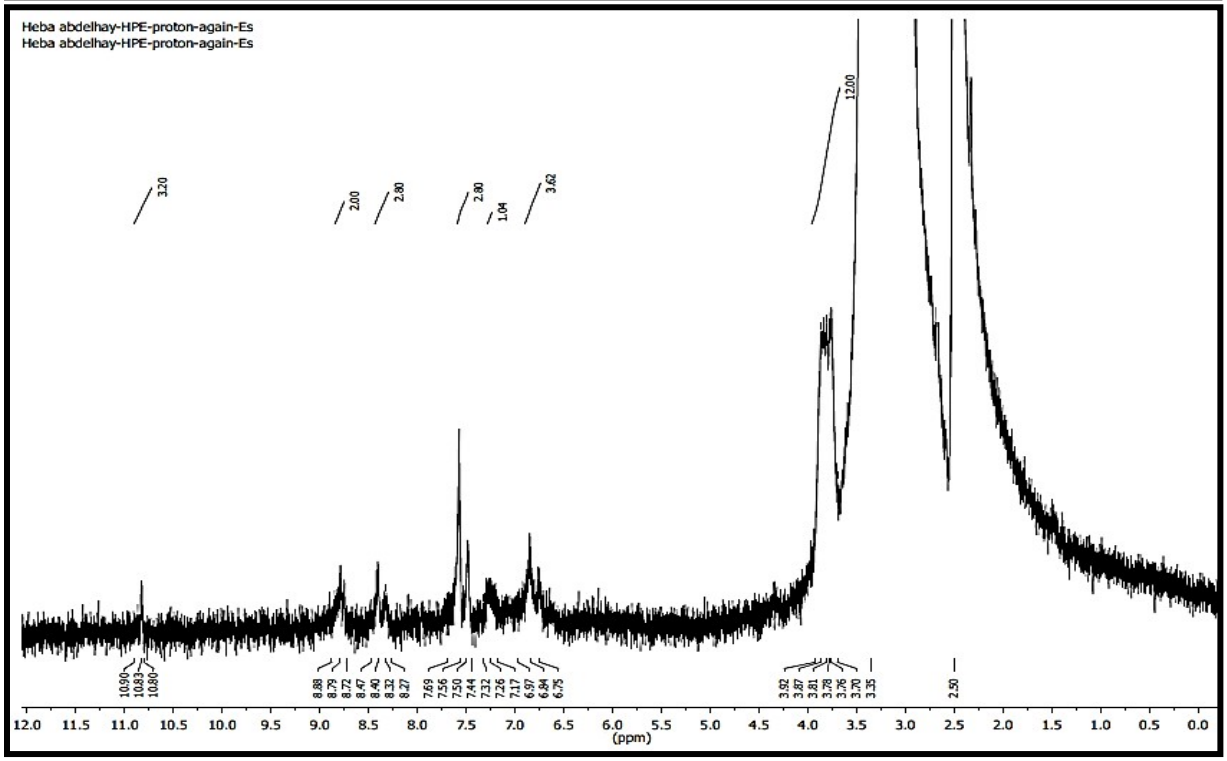


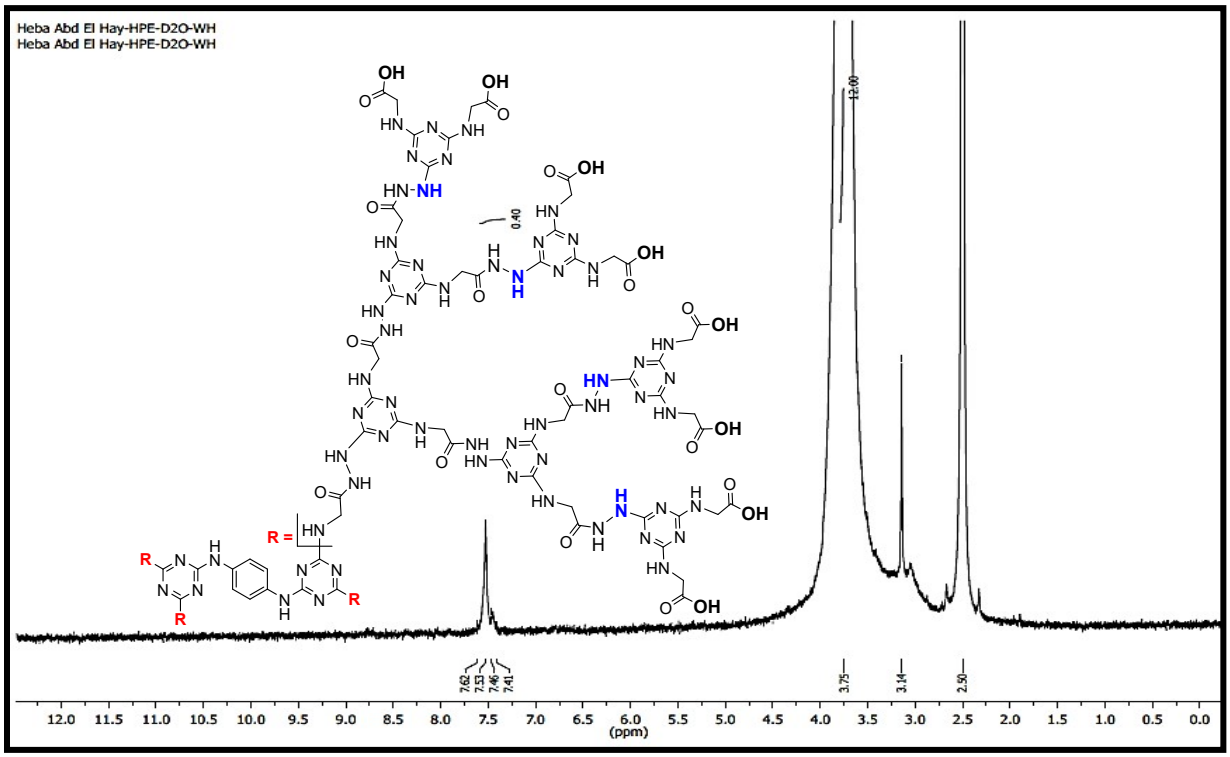


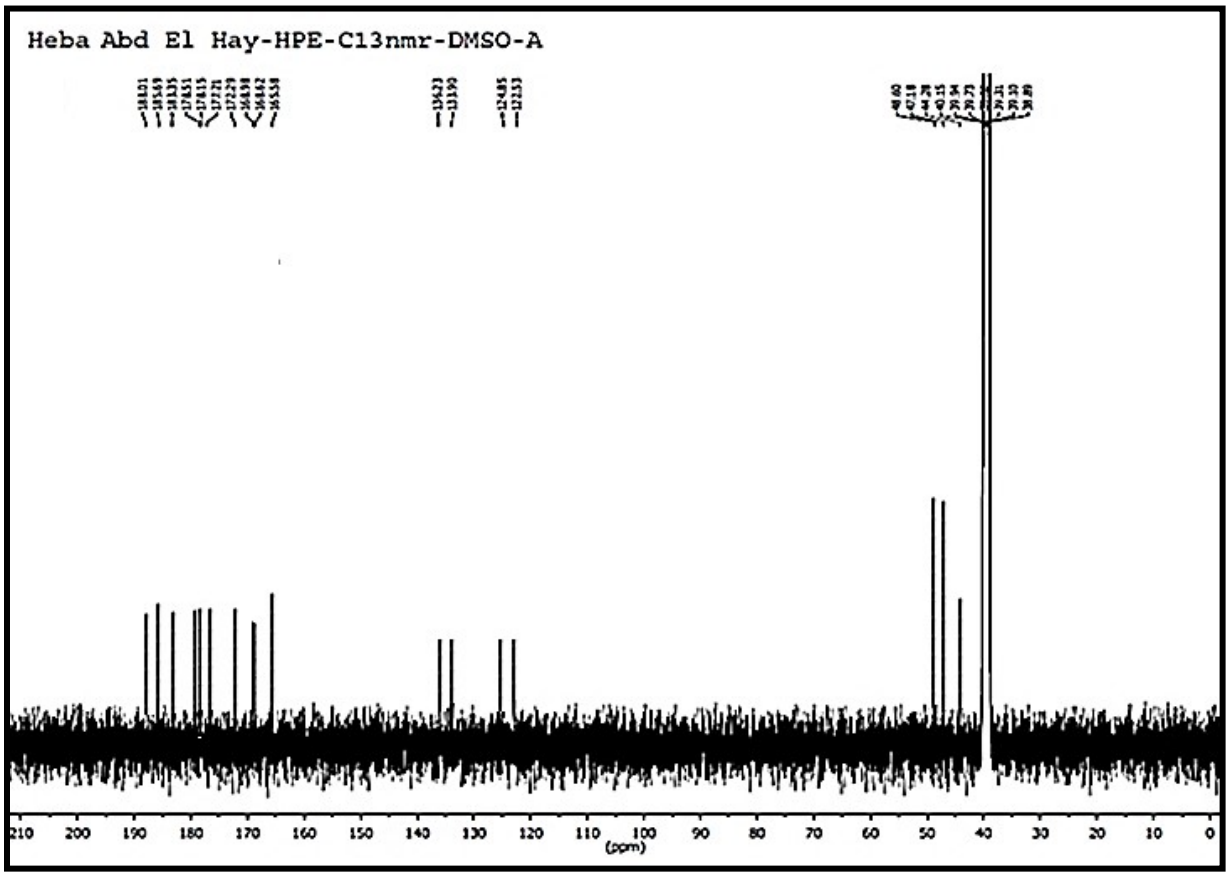

Dr Heba abdelHay_HPT_carbon-1+2-6.jdf
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
(ppm)

S12- IR (KBr), ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (DMSO-d_6) of (TPhyG$_4$-OH) **15**






Heba Abd El Hay-HPE-D2O-WH
Heba Abd El Hay-HPE-D2O-WH
R =
12.00
0.40
7.62
7.53
7.46
7.41
3.75
3.14
2.50
(ppm)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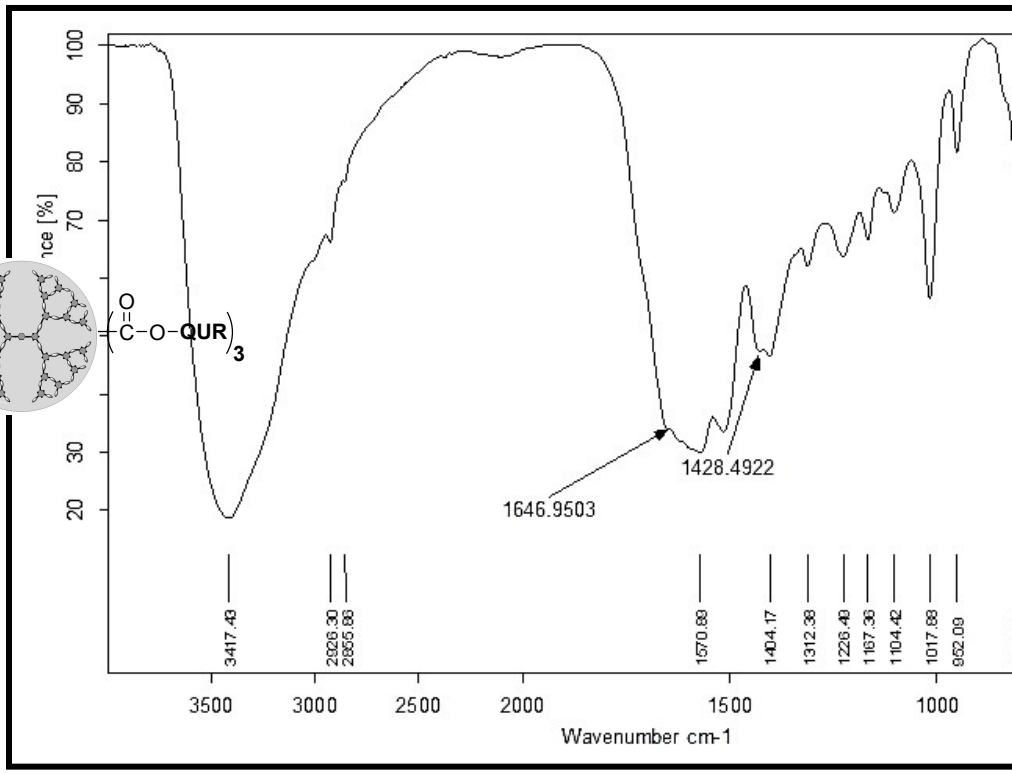
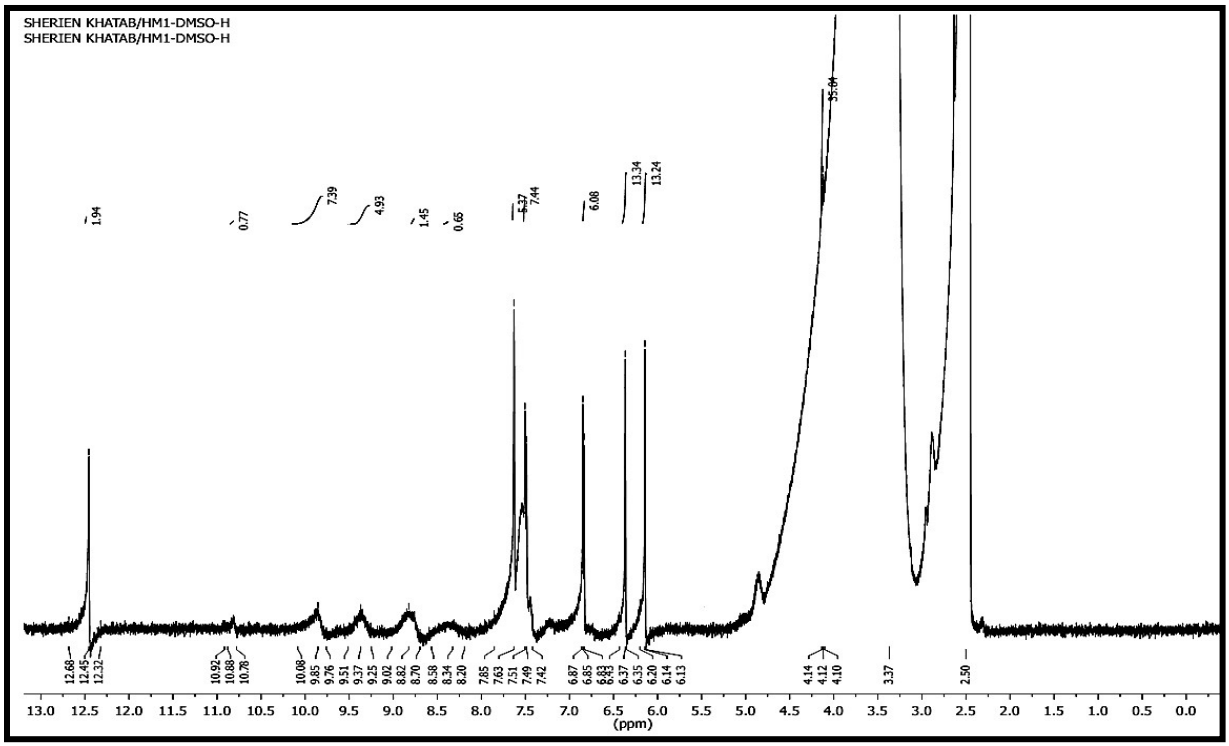
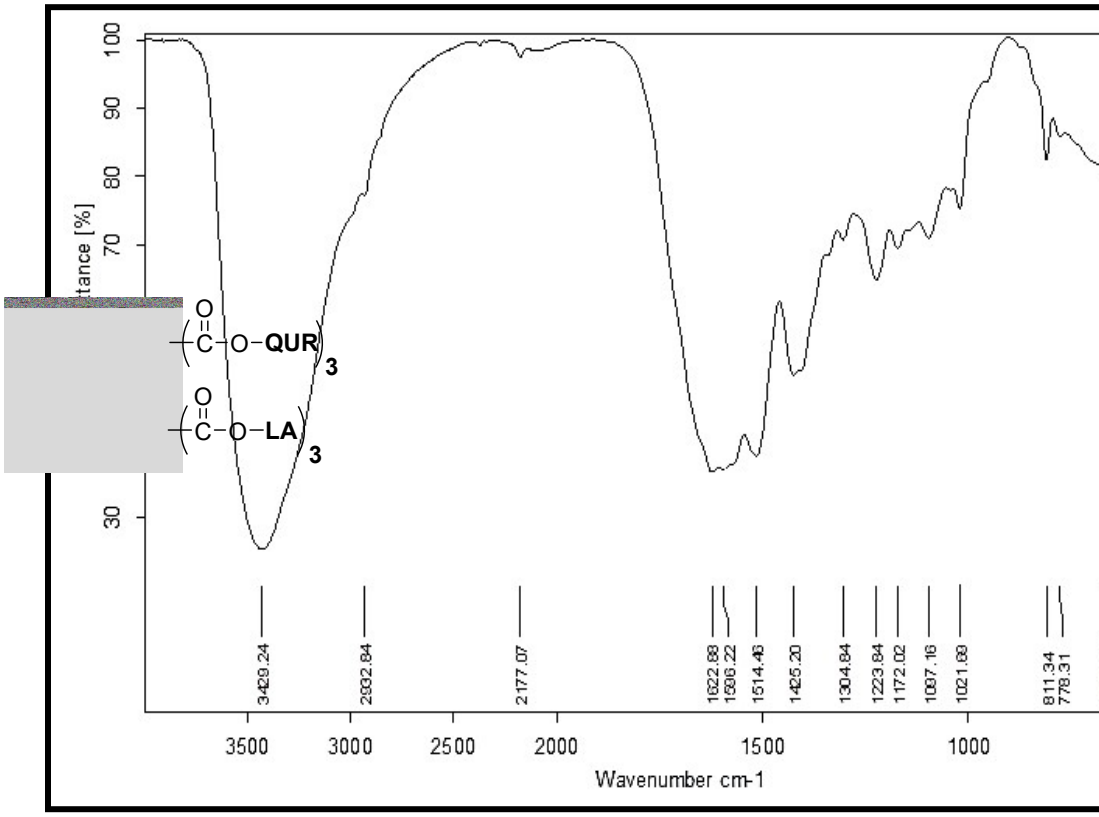
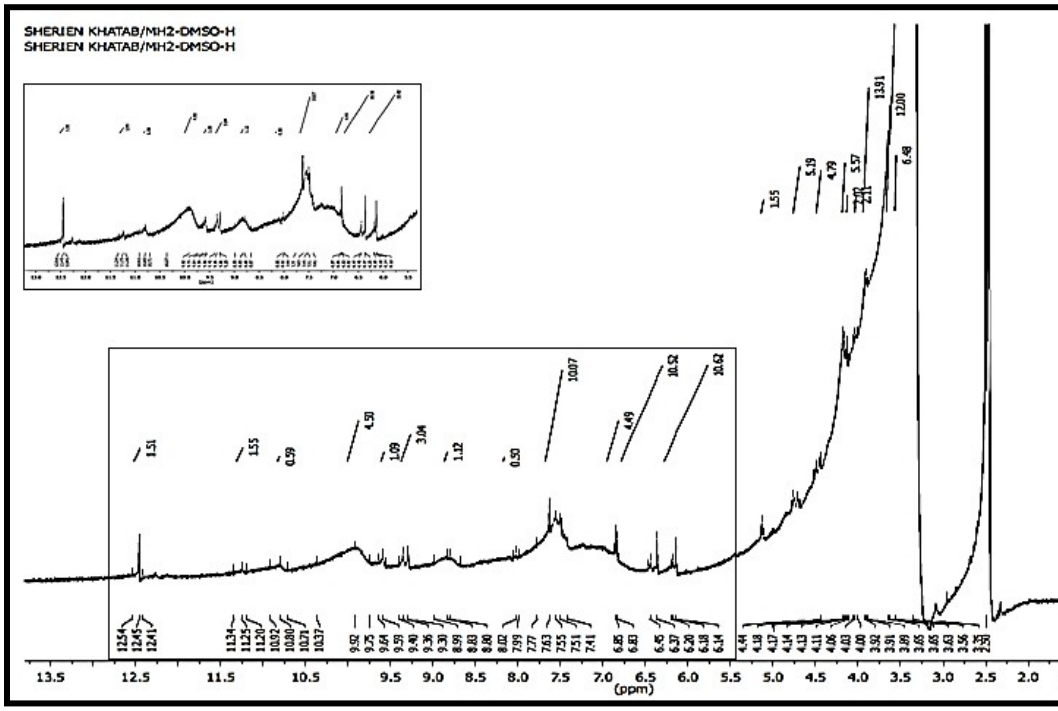
S13- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of (TPhyG$_3$-OH)-QUR **16**

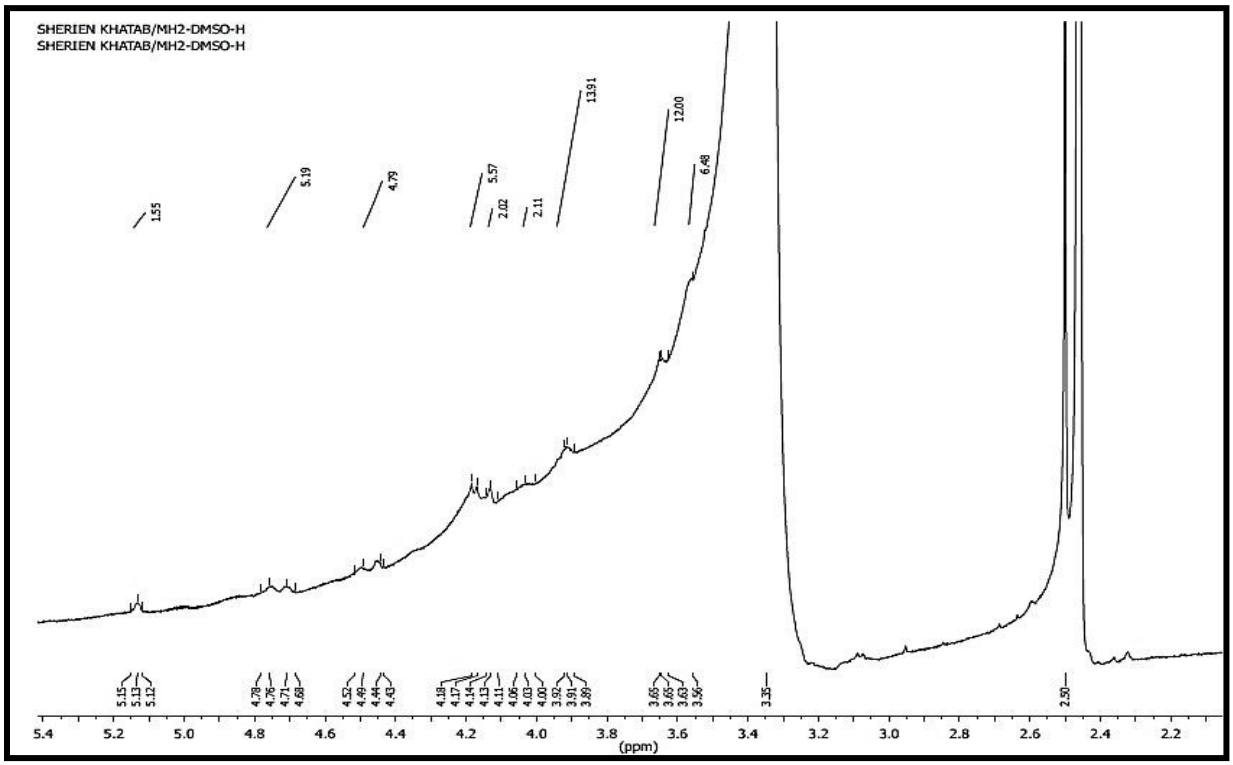




S14- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of (TPhyG$_3$-OH)-QUR-LA **17**



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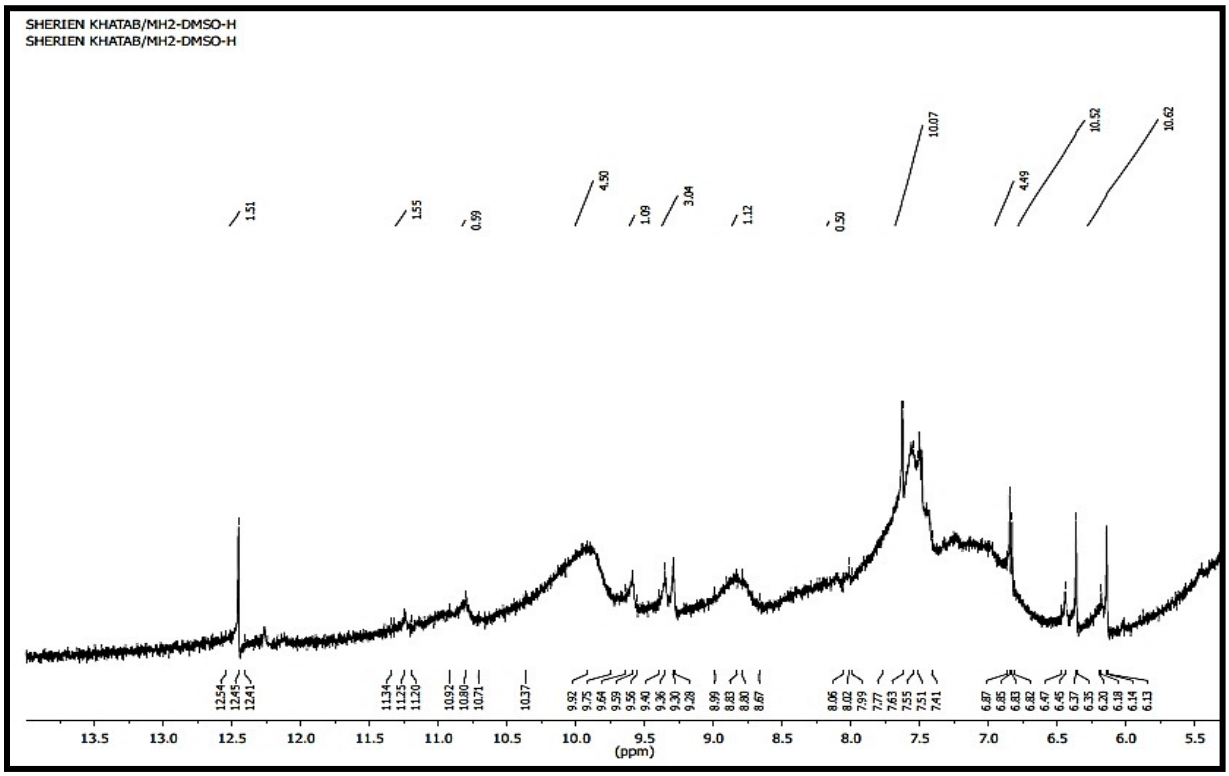
SHERIEN KHATAB/MH2-DMSO-H
(ppm)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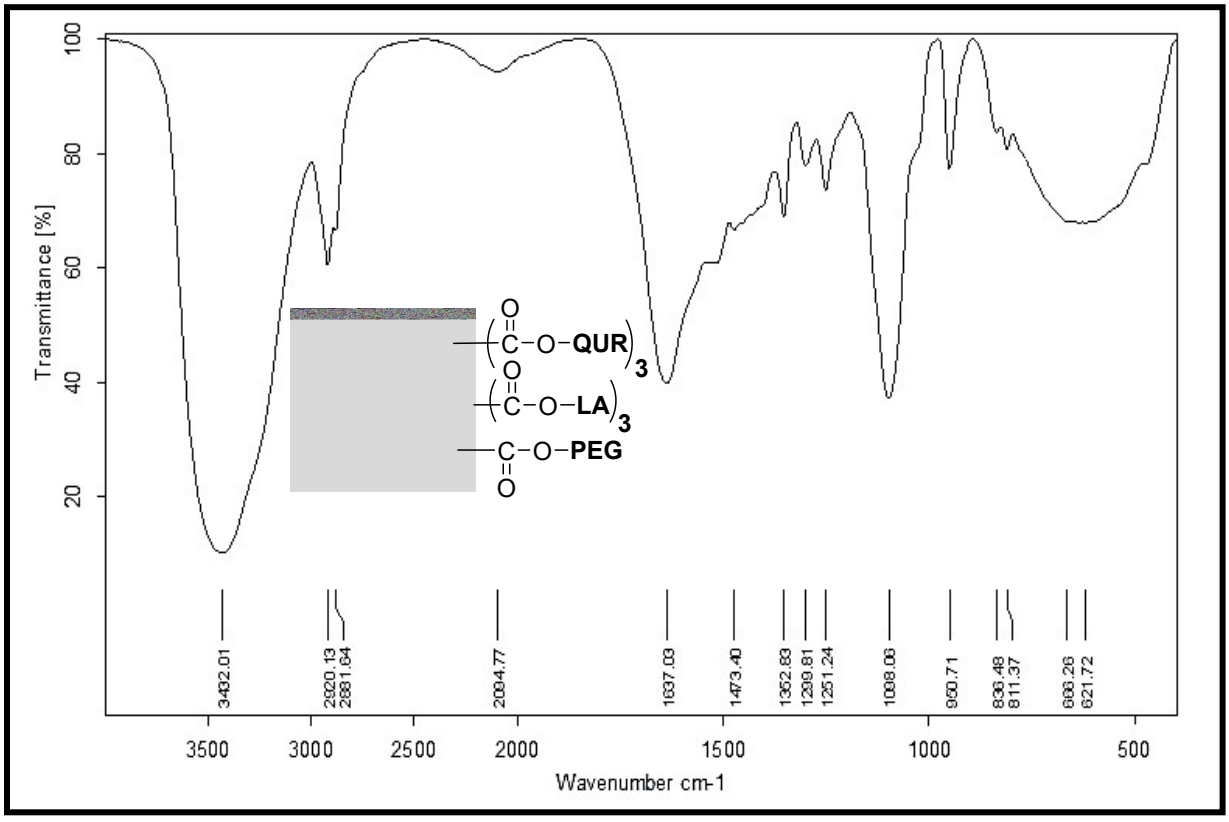
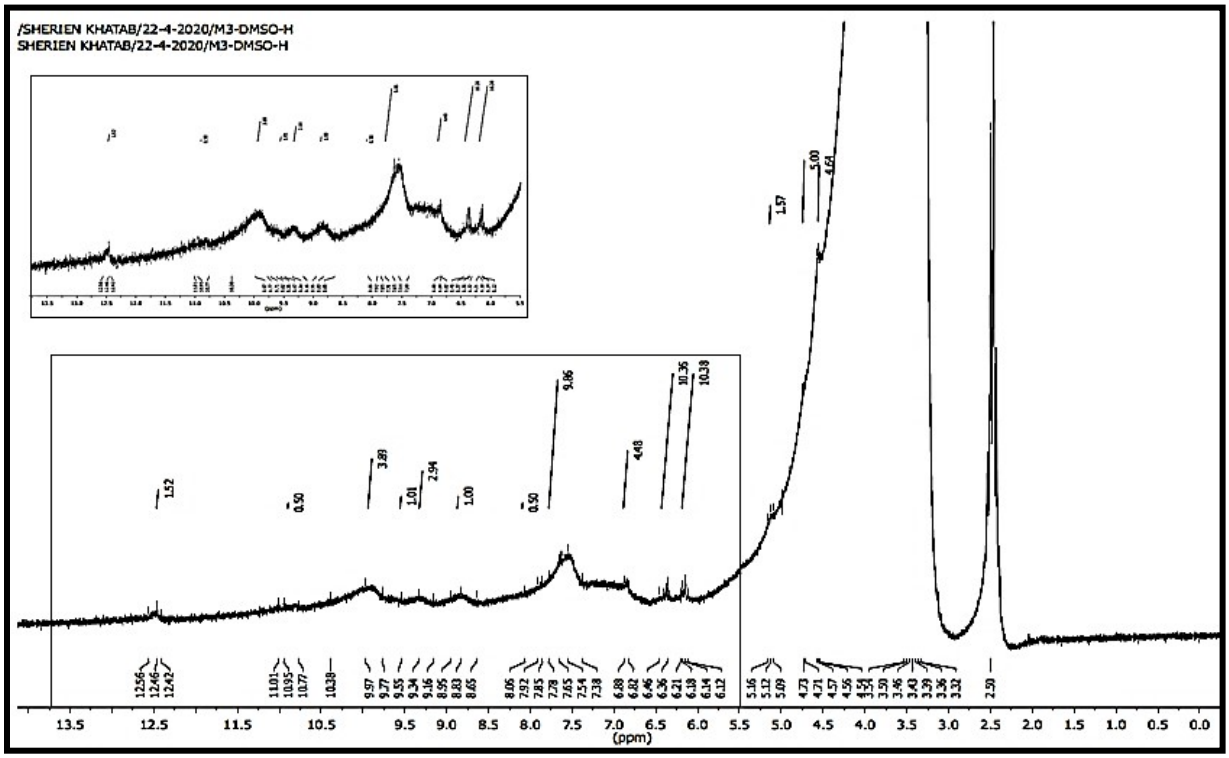
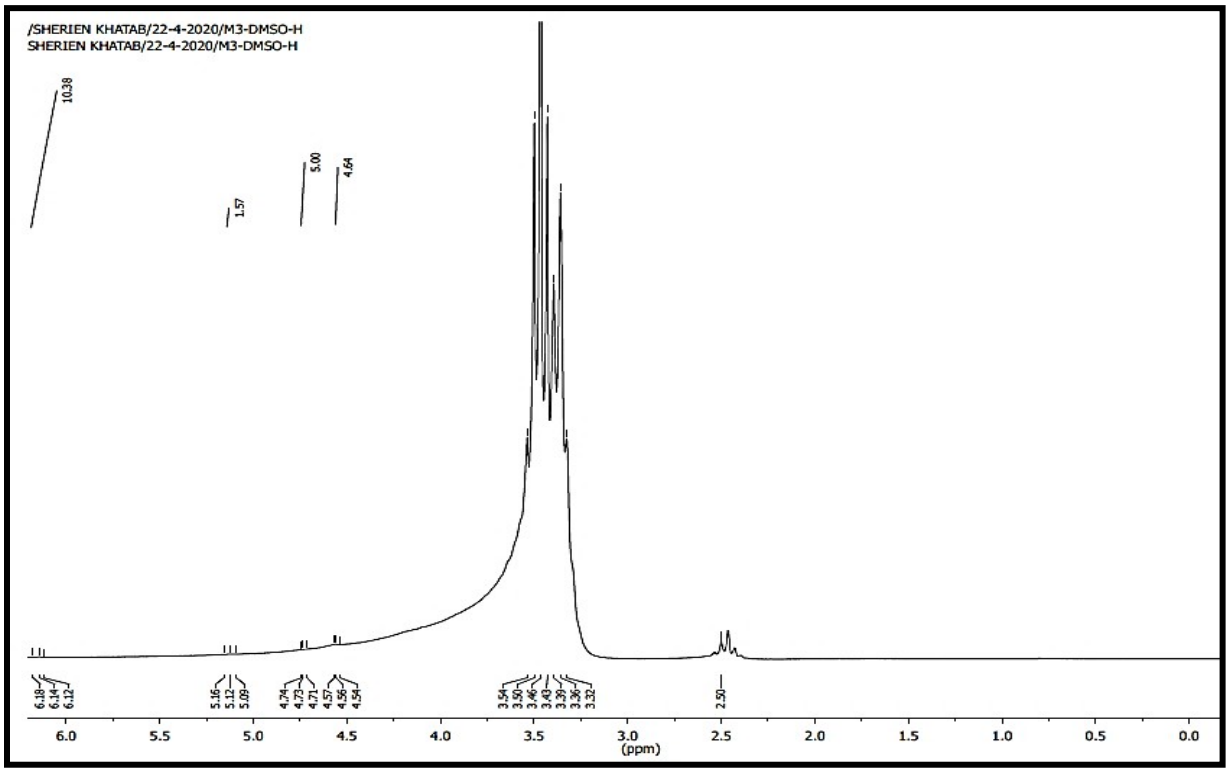
S15- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of (TPhyG$_3$-OH)-QUR-LA-PEG **18**



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3.50
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3.43
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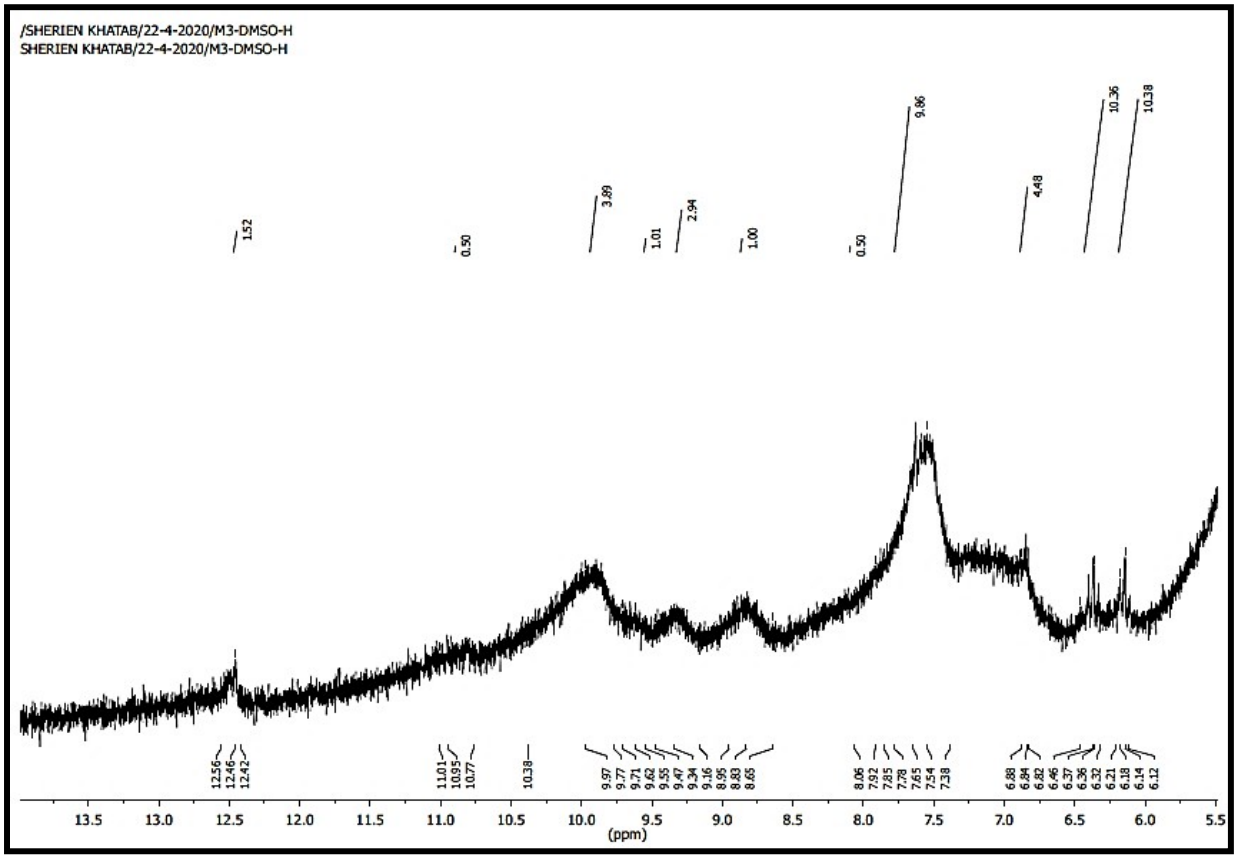
(ppm)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11.01
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9.34
9.16
8.95
8.83
8.65
8.06
7.92
7.85
7.78
7.65
7.54
7.38
6.88
6.84
6.82
6.46
6.37
6.36
6.32
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12.0
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11.0
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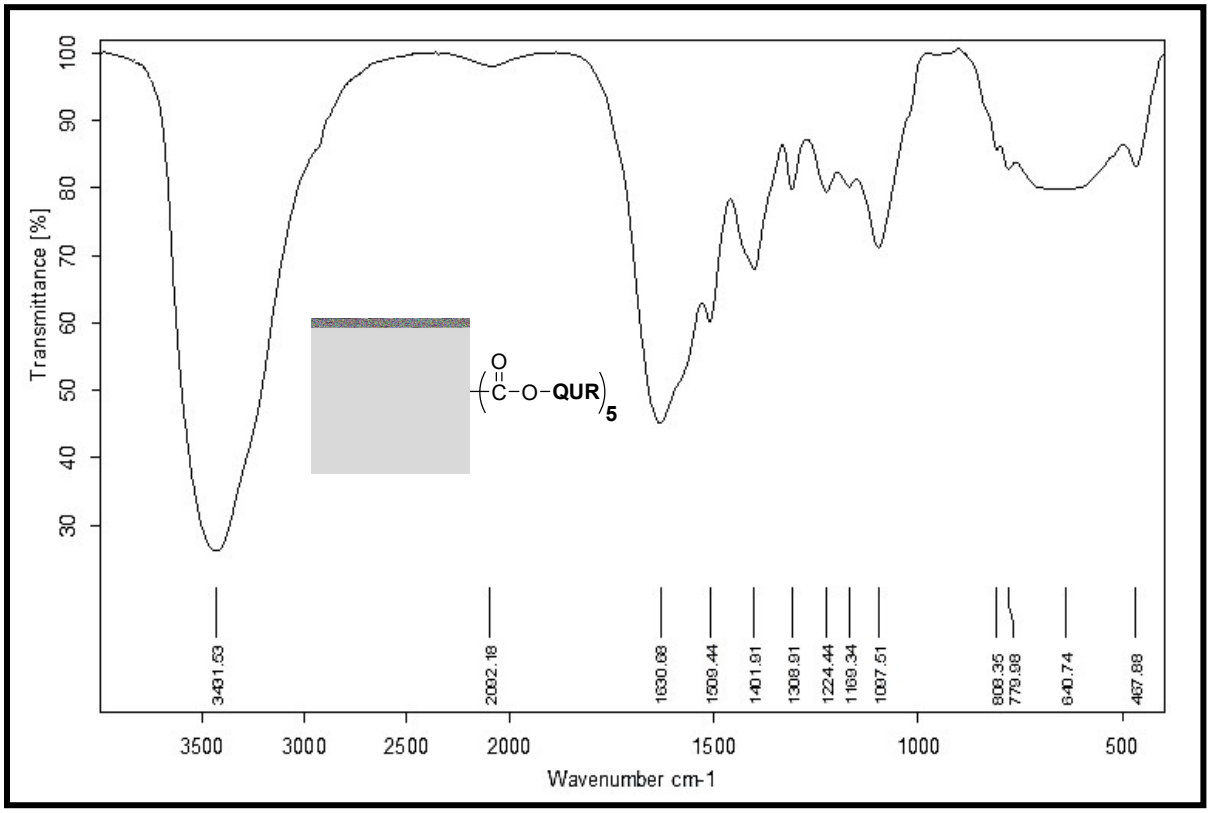
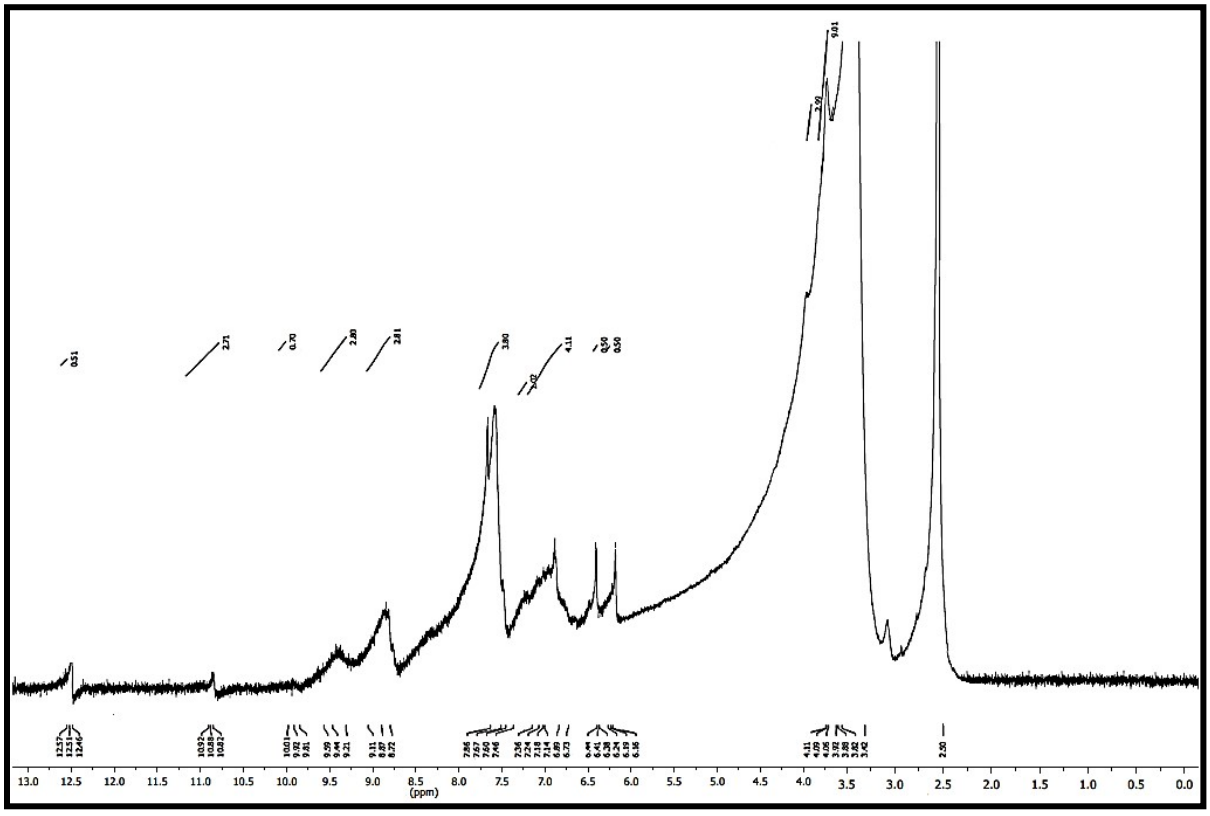
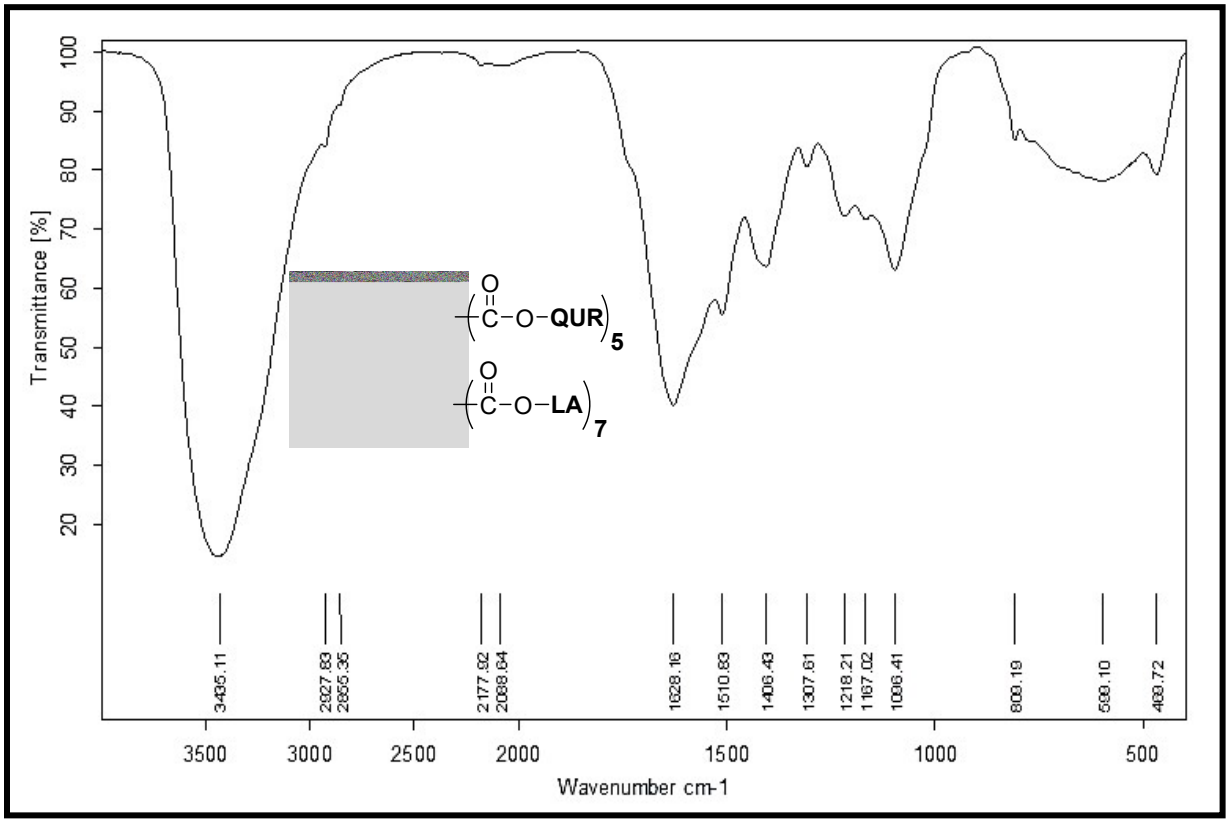
(ppm)

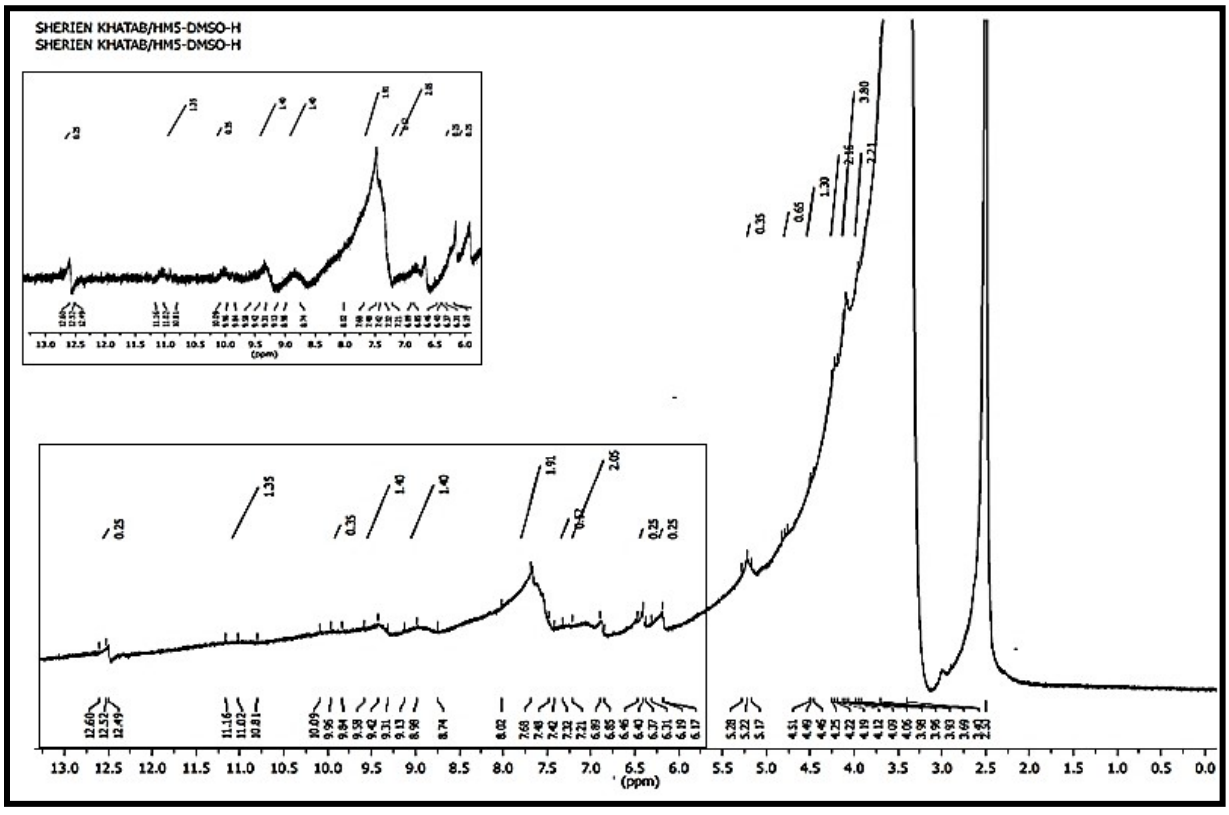
S16- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of (TPhyG$_4$-OH)-QUR **19**



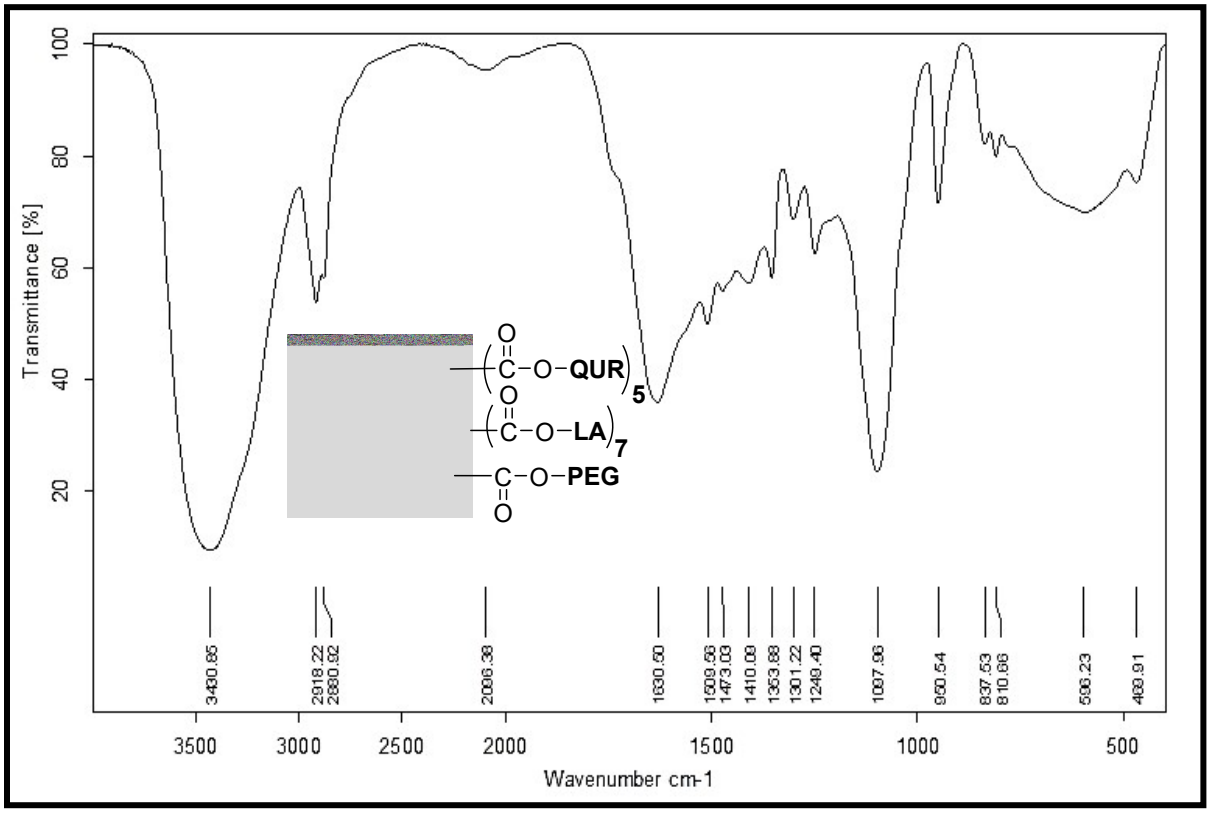


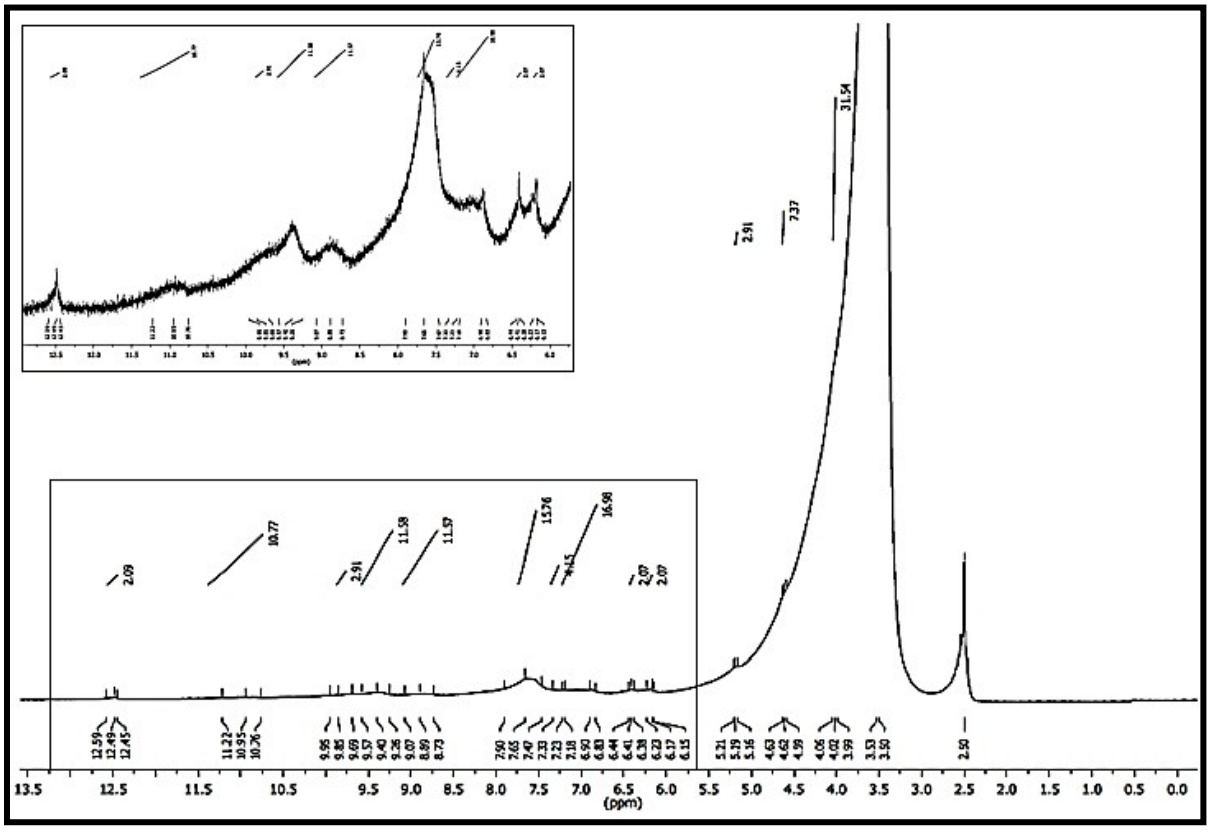
S17- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of (TPhyG$_4$-OH)-QUR-LA **20**

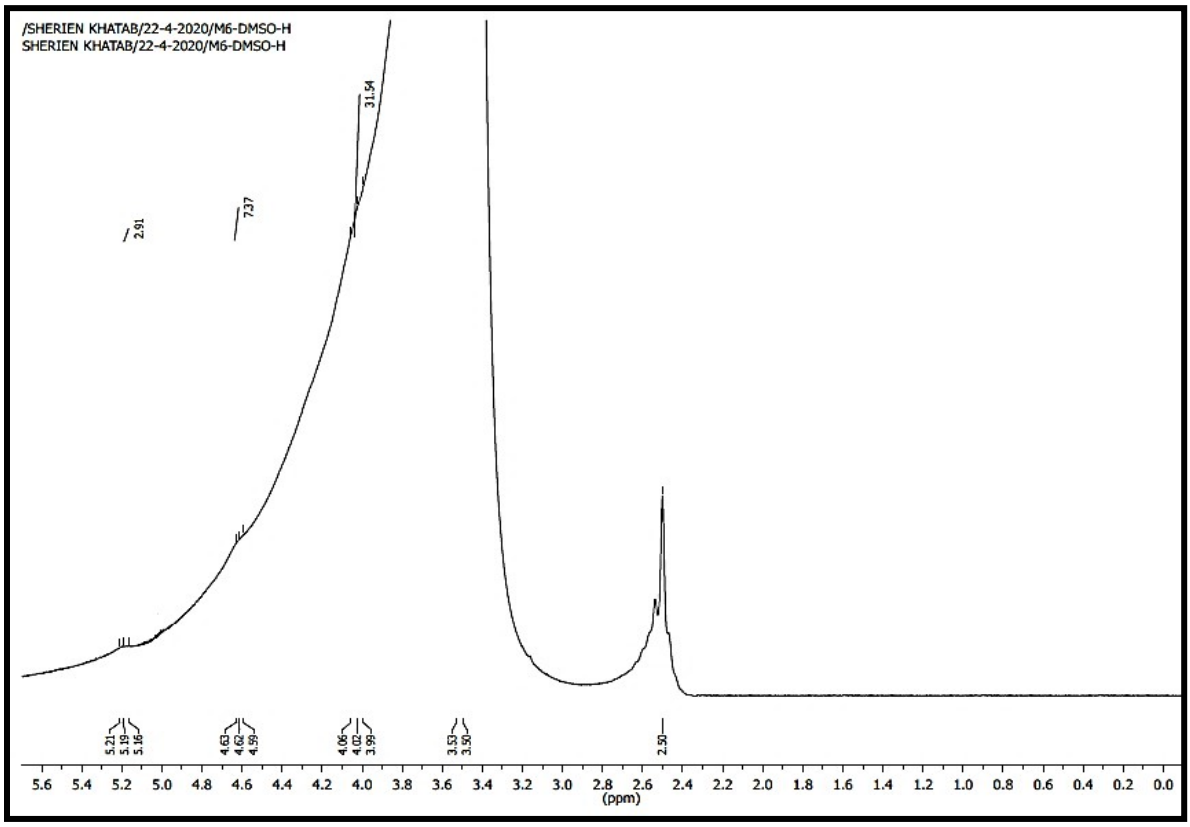




S18- IR (KBr) and ^{1}H-NMR (DMSO-d_6) of (TPhyG$_4$-OH)-QUR-LA-PEG **21**



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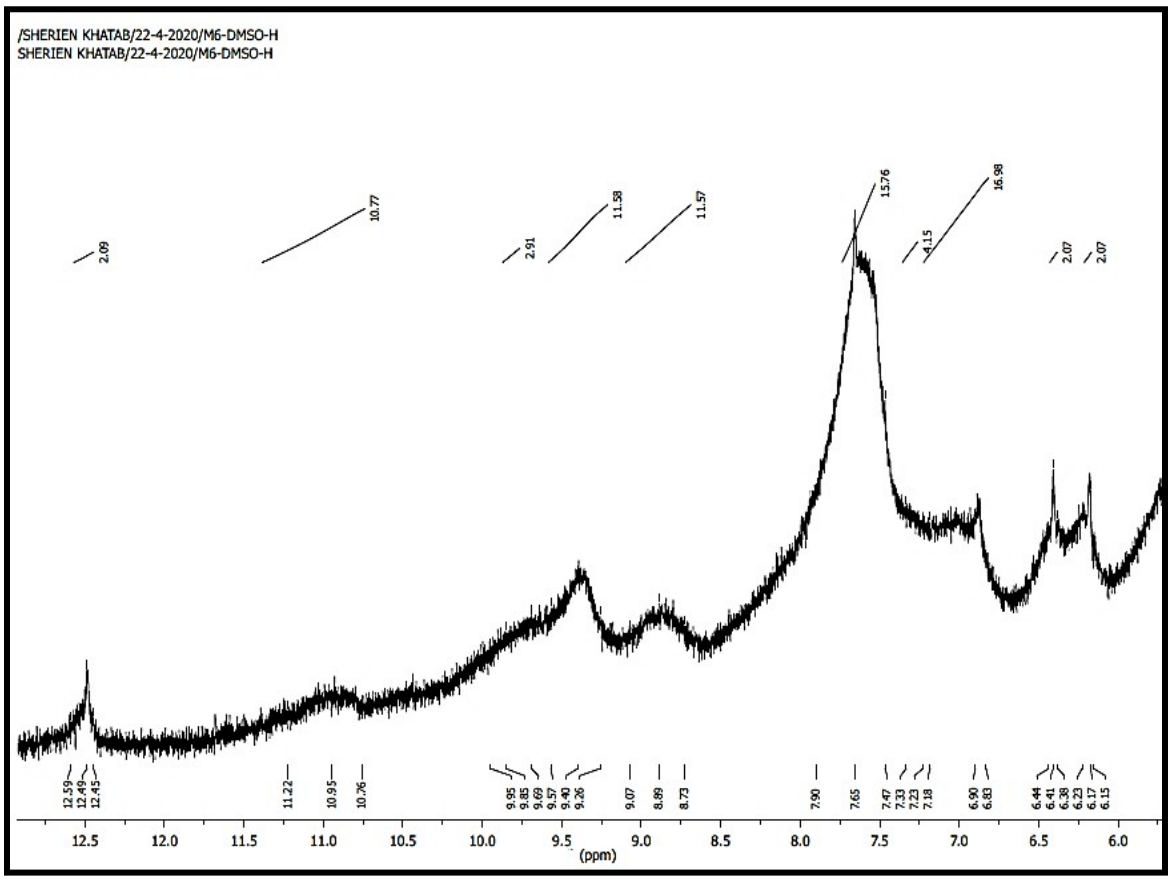
4.06
4.02
3.99
3.53
3.50
2.50
(ppm)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2.07
12.59
12.49
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11.22
10.95
10.76
9.95
9.85
9.69
9.57
9.40
9.26
9.07
8.89
8.73
7.90
7.65
7.47
7.33
7.23
7.18
6.90
6.83
6.44
6.41
6.38
6.23
6.17
6.15
(ppm)

1.1.6. Physicochemical characterization (S19):

- **HPLC Assay of Quercetin (QUR):**

Quantification of Quercetin was performed using the following HPLC method developed and validated in our laboratory.

- **Chromatographic conditions:**

HPLC Agilent 1200 series (quaternary pump, auto-injector, vacuum degasser, diode array and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation Software (Agilent Technologies, Santa Clara, CA, USA). The HPLC analysis was carried out with Agilent technologies Microsorb-MV 100-5 C_{18} column (250 ×4.6 mm and particle size 5 µm) with temperature at 30°C. For quercetin chromatographic elution, the injection volume was 5 µL. The mobile phase consisted of 75%:25% methanol: 0.1% TFA. Total run time was 5 minutes pumped at flow rate 1 mL/min, quercetin was detected at 373 nm with a retention time of 4.2 min.

- **Stock and standard solutions preparation of (QUR) calibration curve:**

For standard solution preparation, 10 mg/mL stock solution of QUR was prepared by dissolving 100 mg of QUR in 10 mL methanol and the serial working standard solutions were prepared in the mobile phase. Samples for the calibration curve and validation assessment were prepared by dilution of the stock solutions of QUR. Quantification of the drug was accomplished by calibration standard curve (peak area versus drug concentration). Calibration curve was linear over the QUR selected concentrations.

- **Method validation and Linearity:**

Summary of linear regression data for the calibration curve is presented in Table 1. Calibration curve was constructed using peak area (y-axis) against analyte concentration (x-axis). The calibration curve consisted of blank sample and at least non-zero four calibration levels covering the method range. All reported concentrations were reported within the calibration range 0.002-1.0 mg mL^{-1} (Figure 1). Retention time and peak performance parameters for quercetin at 0.1 and 0.2 mg mL^{-1} when analyzed by the mentioned chromatographic conditions is presented in (Figures 2 and 3).

Table 1. Summary of linear regression data for calibration curves using peak areas.

Parameters	Linearity range	Linear regression equation	Determination coefficient (r^2)	Slope	Intercept	Limit of Detection	Limit of Quantification
Quercetin	0.002-1.0 mg mL^{-1}	y=17879x	0.99998	17879.32	4.9348	0.0072	0.0218

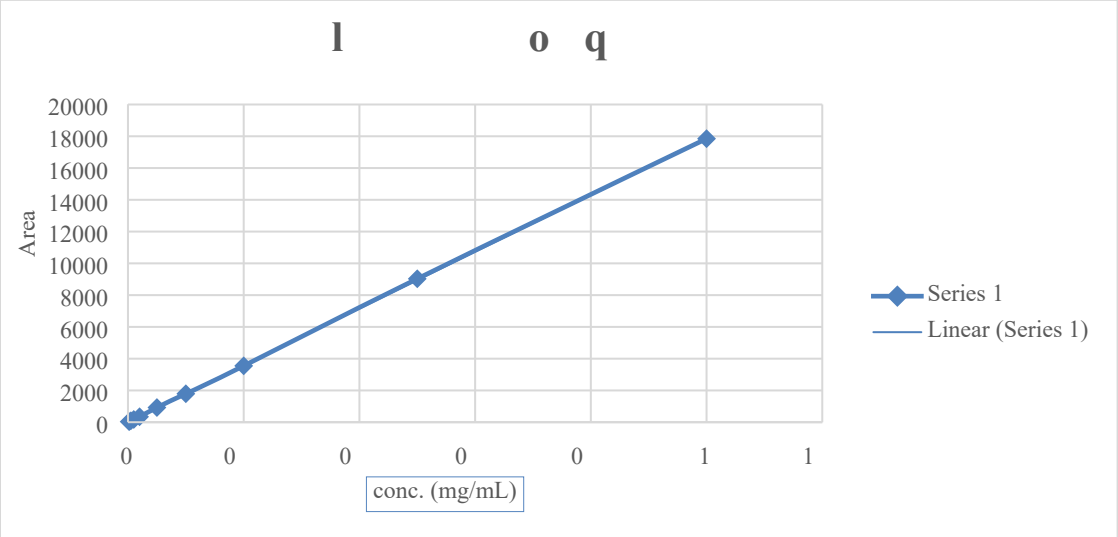

Figure 1. Standard calibration curve of quercetin in methanol at λ=373 nm.

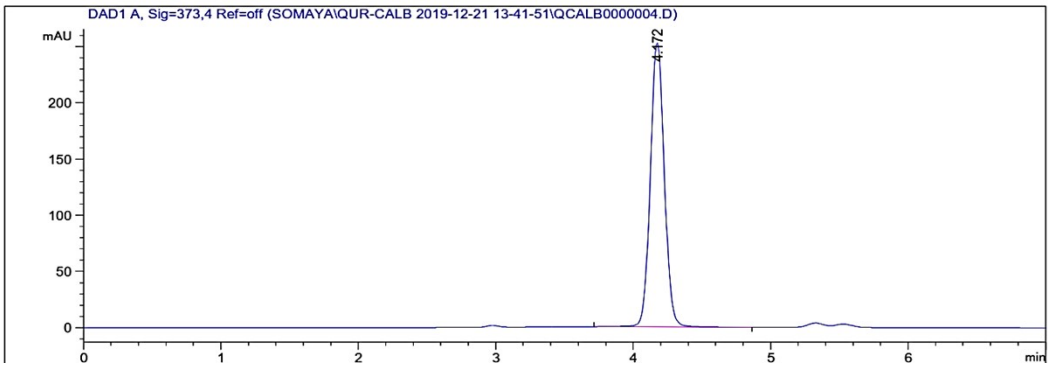

Figure 2. Retention time and peak performance parameters for quercetin at 0.1 mg/mL when analyzed by the mentioned chromatographic conditions.

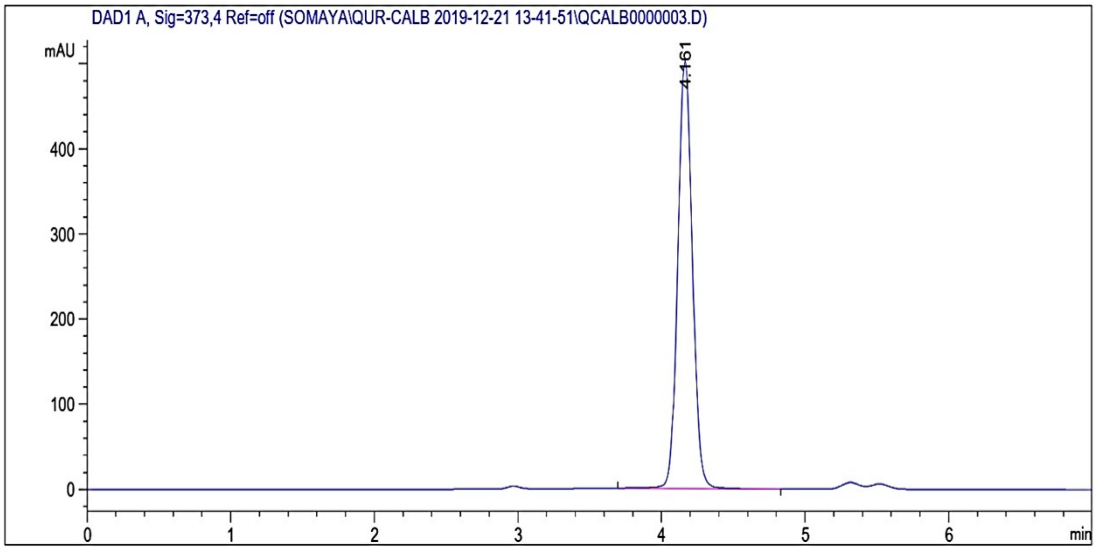

Figure 3. Retention time and peak performance parameters for quercetin at 0.2 mg/mL when analyzed by the mentioned chromatographic conditions.

- **Drug content:**

The conjugation efficiency of QUR in ((TPhyG_3-OH)-QUR **16**, (TPhyG_3-OH)-QUR-LA **17**, (TPhyG_3-OH)-QUR-LA-PEG **18**, (TPhyG_4-OH)-QUR **19**, (TPhyG_4-OH)-QUR-LA **20** and (TPhyG_4-OH)-QUR-LA-PEG **21** were determined indirectly from the difference between the amount of free drug in the dialysate and the total added drug amount. After each dialysis, the dialysate was obtained and analyzed for the drug using our developed HPLC method for the determination of QUR as shown in Table 2.

$$\text{Conjugation \%} = \frac{\text{Amount of the initial QUR- Amount of QUR in the supernatants}}{\text{Amount of the initial QUR}} * 100 \quad \text{(Eq. 1)}$$

- **Particle size and zeta potential:**

Particle size (PS) was measured by Zetasizer Nano ZS /ZEN3600 (Malvern, Instruments Ltd, Malvern, UK). The PS was measured with the non-invasive backscattering technology at a detection angle of 173° after dilution with purified water to an appropriate concentration. All the DLS measurements were performed at 25.0±0.1°C for three repeated measurements. The zeta potential of the prepared nanoparticles (NPs) each diluted NPs suspension (1 mL) was put in a universal folded capillary cell equipped with platinum electrodes and was estimated following the same method of that used for measuring zeta potential.

2. Biology

2.1. Cytotoxicity evaluation (S20)

Wi-38 cell line was cultured in DMEM medium-contained 10% fetal bovine serum (FBS), seeded as $5x10^3$ cells per well in 96-well cell culture plate and incubated at 37ºC in 5% CO_2 incubator. After 24 h for cell attachment, serial concentrations of dendrimers ((TPhyG_3-OH) **12** and (TPhyG_4-OH) **15**), and dendrimer drug conjugates ((TPhyG_3-OH)-QUR, (TPhyG_3-OH)-QUR-LA, (TPhyG_3-OH)-QUR-LA-PEG, (TPhyG_4-OH)-QUR, (TPhyG_4-OH)-QUR-LA, (TPhyG_4-OH)-QUR-LA-PEG) were incubated with Wi-38 cells for 72 h. Cell viability was assayed by MTT method.[4] Twenty microliters of 5 mg/mL MTT (Sigma, USA) was added to each well and the plate was incubated at 37ºC for 3 h. Then MTT solution was removed, 100 µL DMSO was added, and the absorbance of each well was measured with a microplate reader (BMG LabTech, Germany) at 570 nm. The effective safe concentration (EC_{100}) value (at 100% cell viability) of the tested compounds was estimated by the Graphpad Instat software.

2.2. *In vitro* MMP-2/9 inhibition (S21)

MMP-2 inhibition assay protocol

- Add test compounds and diluted enzyme solution to the microplate wells.
- For one well of 96-well plate, the suggested volume of enzyme solution is 40 µL and 10 µL of test compound.
- Simultaneously establish the following control wells.
- Vehicle control contains MMP-2 enzyme and vehicle used in delivering test compound (DMSO, concentration not to exceed 1%).
- bring the total volume of all controls to 50 µL. Pre-incubate the plate for 10 min. at assay temperature to 37°C.
- Run the enzymatic reaction. Add 50 µL of MMP-2 substrate solution into each well. Mix the reagents completely by shaking the plate gently for 30 sec.
- Incubate the reaction for 60 min. Add 50 µL of stop solution to each well. Mix the reagents and measure absorbance at 412 nm.
- Inhibitory activity was expressed as IC_{50} values (the concentration at which 50 % of the enzyme activity inhibited), which were calculated from dose-response curve

obtained using eight tested concentrations of the inhibitor and carried out in duplicate (**Figure 4**).

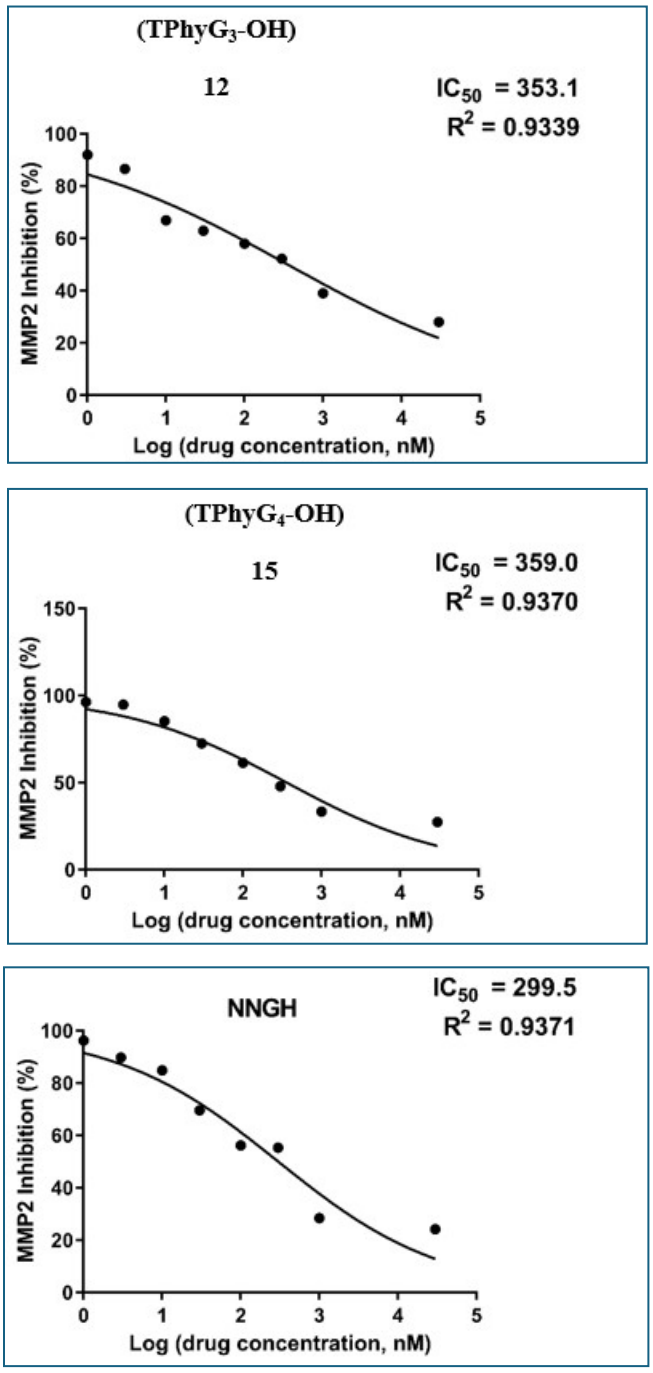

Figure 4. Dose-response curve for MMP-2 inhibition versus serial dilutions of compounds **12, 15** and NNGH.

MMP-9 inhibition assay protocol

- Briefly warm kit components MMP Substrate and MMP Inhibitor to RT to thaw DMSO.
- Dilute MMP inhibitor 1/200 in Assay Buffer as follows. Add1 µL inhibitor into 200 µL Assay Buffer, in a separate tube. Warm to reaction temperature (e.g., 37°C).
- Dilute MMP9 substrate 1/25 in assay buffer to required total volume (10 µL are needed per well). For example, for 15 wells dilute 6.4 µL MMP substrate into 153.6 µL Assay Buffer, in a separate tube. Warm to reaction temperature (e.g., 37°C).
- Dilute MMP9 enzyme 1/60 in Assay Buffer to required total volume (20 µL are needed per well). Warm to reaction temperature (e.g., 37°C) shortly before assay.
- Pipette assay buffer into each desired well of the 1/2 volume microplate as follows:
  Blank (no MMP-9) =90 µL Assay Buffer
  Control (no inhibitor) =70 µL Assay Buffer
  MMP Inhibitor =50 µL Assay Buffer
  Test inhibitor=varies
- Allow microplate to equilibrate to assay temperature (e.g., 37°C).
- Add 20 µL MMP-9 (diluted in step 4) to the control, MMP Inhibitor, and test inhibitor wells. Final amount of MMP-9 will be 0.9 U per well (9 mU/µL). Remember to not add MMP-9 to the blanks!
- Add 20 µL MMP Inhibitor (diluted in step 2) to the MMP Inhibitor wells only! Final inhibitor concentration=1.3 µM.
- Add desired volume of test inhibitor to appropriate wells.
- Incubate plate for 30-60 minutes at reaction temperature (e.g., 37°C) to allow inhibitor/enzyme interaction.
- Start reaction by the addition of 10 µL MMP9 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration=100 µM.
- Continuously read plates at A412nm in a microplate reader. Record data at 1 min. time intervals for 10 to 20 min.
- Perform data analysis, IC_{50} values were calculated from dose-response curve obtained using different tested concentrations of the inhibitor (**Figure 5**).

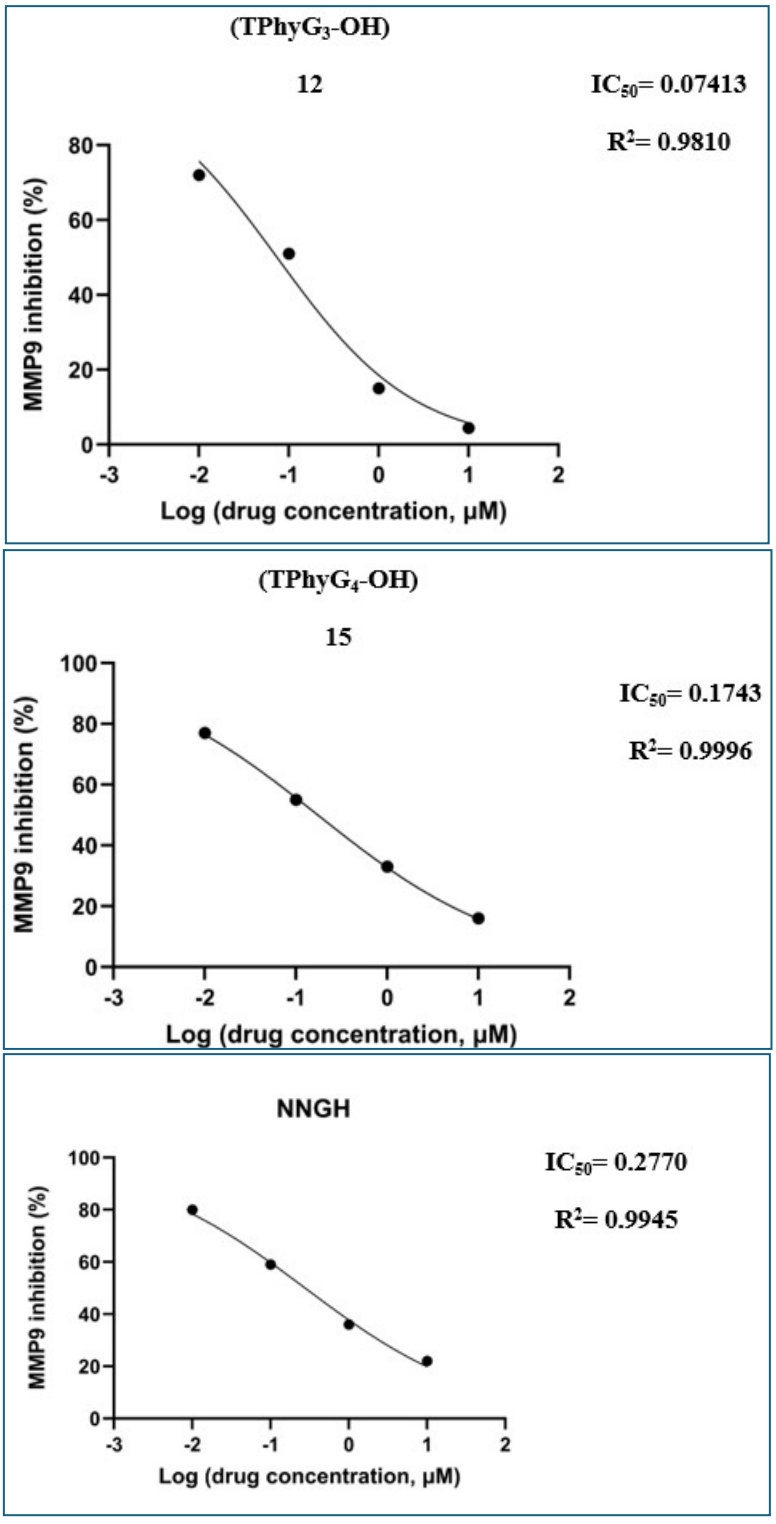

Figure 5. Dose-response curve for MMP-9 inhibition versus serial dilutions of compounds **12, 15** and NNGH.

2.3. Anticancer evaluation (S22):

Anticancer activities of the synthesized dendrimers ((TPhyG$_3$-OH) **12** and (TPhyG$_4$-OH) **15**), and dendrimer drug conjugates ((TPhyG$_3$-OH)-QUR, (TPhyG$_3$-OH)-QUR-LA, (TPhyG$_3$-OH)-QUR-LA-PEG, (TPhyG$_4$-OH)-QUR, (TPhyG$_4$-OH)-QUR-LA, (TPhyG$_4$-OH)-QUR-LA-PEG) were evaluated on breast cancer cell line (MCF-7 cells) and hepatocellular carcinoma (HepG-2) cell line in comparison to reference chemotherapy (**Figures 6** and **7**). Cancer cells were cultured in RPMI-1640 (Lonza, USA) supplemented with 10% FBS. All cancer cells (4x10^3 cells/well) were seeded in sterile 96-well plates. After 24 h, serial concentrations of the tested compounds were incubated with the cancer cell lines for 72 h at 37ºC in 5% CO_2 incubator. MTT method was done as described above. The half maximal inhibitory concentration (IC_{50}) values were calculated using the Graphpad Instat software. Furthermore, cellular morphological changes before and after treatment with the most effective and safest anticancer compounds were investigated using phase contrast inverted microscope with a digital camera (Olympus, Japan).

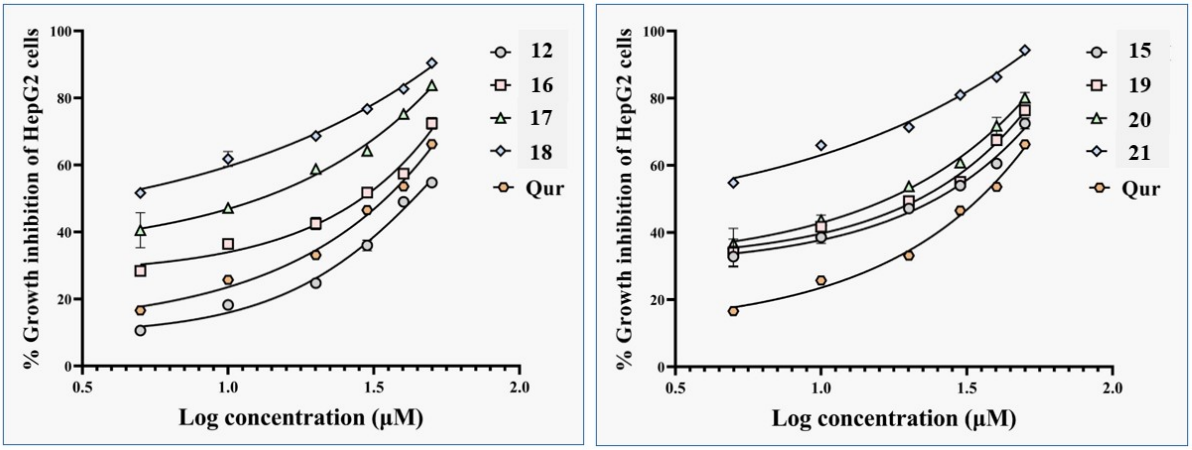

Figure 6: Growth inhibition curves of tested dendrimers conjugates and quercetin against HepG-2 cells.

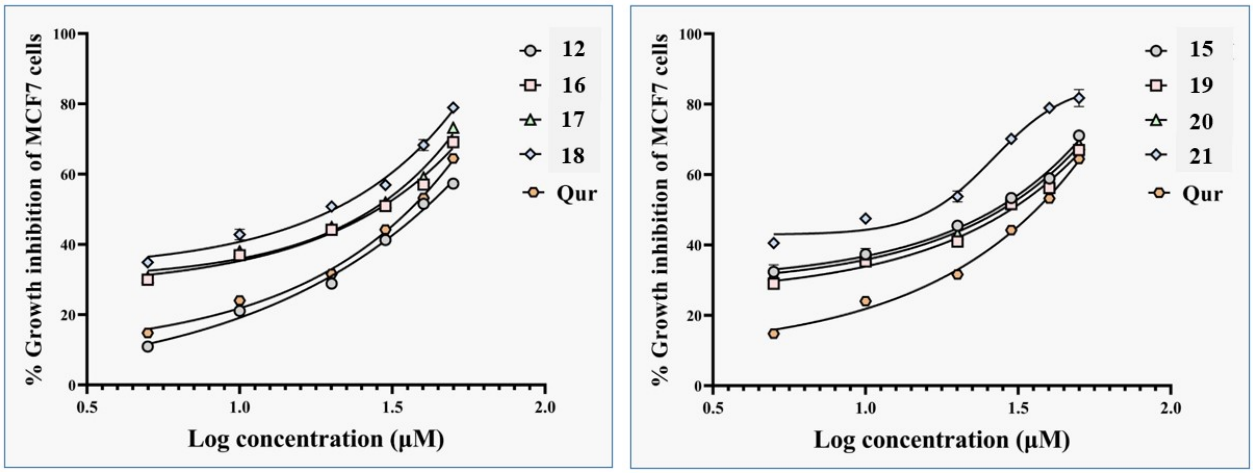

Figure 7: Growth inhibition curves of tested dendrimers conjugates and quercetin against MCF-7 cells.

2.4. Flow cytometric analysis of apoptotic effects of the most active and safe compounds (S23):

Dendrimer drug conjugates (TPhyG$_3$-OH)-QUR, (TPhyG$_3$-OH)-QUR-LA, (TPhyG$_3$-OH)-QUR-LA-PEG, (TPhyG$_4$-OH)-QUR, (TPhyG$_4$-OH)-QUR-LA, (TPhyG$_4$-OH)-QUR-LA-PEG) were incubated, for 72 h, with the cancer cells. After trypsinization, the untreated and treated cells were incubated with annexin V/PI for 15 min. Then cells were fixed and incubated with streptavidin-fluorescein (5 µg/mL) for 15 min. The apoptosis-dependent anticancer effect was determined by quantification of annexin-stained apoptotic cells using the FITC signal detector (FL1) against the phycoerythrin emission signal detector (FL2).

3. Docking simulation (S24):

MOE 2019.102.[5] was employed for performing docking studies. The structural coordinates of MMP-9 catalytic domain complexed with a reverse hydroxamate inhibitor were downloaded from the protein data bank (PDB ID: 1GKC [6]). Unwanted residues were removed. The protein structure was prepared and refined utilizing the default settings of the MOE "structure preparation" module. The active dendrimer **12** was

built *in silico* and energy minimized employing Amber10:EHT force field with reaction-field electrostatics (an interior dielectric constant of 1 and an exterior dielectric of 80) using an 8–10 Å cutoff distance. Then docking was conducted into the co-crystallized ligand binding site with various fitting protocols for validation. Rigid docking protocol was adopted. The ligand placement method was set to apply the Triangular matcher algorithm and Alpha HB scoring function as the default scoring function generating the top 10 nonredundant poses of the lowest binding energy conformers of the studied dendrimer for investigation**.**

References

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