Electronic Supplementary Material (ESI) for MedChemComm.

Rationally Synthesized Coumarin Based Pyrazolines ameliorates carrageenan induced inflammation through COX-2/Pro-inflammatory cytokine inhibition

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SUPPORTING INFORMATION

General

Melting points of the synthesized compounds were determined in capillaries. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Advance 400 MHz spectrometers using $CDCl_3$ as solvent. Chemical shifts are stated as δ values in parts per million (ppm) and coupling constants (*J*) given in hertz(Hz). Mass spectra were determined from a Bruker micrOTOF Q II Mass spectrometer. Starting materials and reagents used in reactions were purchased from Sigma Aldrich, TCI and SD fine. Solvents such as methanol, ethanol, hexane, ethyl acetate were distilled prior to use, and stored over calcium hydride or molecular sieves.

General Procedures and Characterization data of Synthesized compounds

General procedure for the preparation of 3-acetyl coumarin (4)[1]

The 3-acetyl coumarin **4** was synthesized according to the literature procedure.[1] To the mixture of salicylaldehyde (**2,** 1eq) and ethyl acetoacetate (**3,** 1eq), few drops of piperidine was added and stirred for 5 minutes at room temperature. The reaction was quenched by neutralization with HCl (1M) and the precipitated product was isolated by filtration. The final compound was then purified by recrystallization in ethanol.

Pale yellow powder, R_f = 0.42, Mp 118-122^{o}C (Lit. 120-122^{o}C), Yield: 89 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.43 (s, 1H, Ar-H), 7.69 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.46-7.30 (m, 3H, Ar-H), 2.31 (s, 3H, CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 175.20, 159.32, 154.24, 137.56, 132.13, 128.45, 126.67, 125.16, 118.71, 116.34, 24.58; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{11}H_8O_3$: 188.0473, Obsd 188.0468.

General procedure for the preparation of Coumarin-Chalcones (6)

To the solution of 3-acetyl coumarin (**4**, 1g) and benzaldehyde (**5**, 1eq) in acetic acid (10 ml), 5-6 drops of conc. H_2SO_4 acid was added slowly and stirred at room temperature for 18-20 h. The reaction was monitored with TLC and then reaction mixture was poured into the cold water. The product precipitated was filtered and recrystallized with methanol.

(E)-3-(3-(4-Methoxyphenyl)acryloyl)-2H-chromen-2-one **(6a)**

Light brown, R_f = 0.44, Yield: 92 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.43 (s, 1H, Ar-H), 7.97 (d, 1H), 7.60 (d, 1H, Ar-H), 7.58 (d, 2H, Ar-H), 7.52 (d, 1H), 7.42-7.30 (m, 3H, Ar-H), 6.84 (d, 2H, Ar-H), 3.75 (s, 3H, OCH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.22, 159.46, 159.37, 154.05, 145.62, 142.33, 135.16, 129.94, 128.28, 127.21, 126.50, 124.80, 124.46, 117.74, 117.13, 112.59, 56.42; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{19}H_{14}N_2O_4$: 306.0892, Obsd 306.0896.

(E)-3-(3-(3,4,5-Trimethoxyphenyl)acryloyl)-2H-chromen-2-one **(6b)**

Pale Yellow, R_f = 0.41, Yield: 94 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.44 (s, 1H, Ar-H), 7.97 (d, 1H), 7.62 (d, 1H, Ar-H), 7.48 (d, 1H), 7.44-7.30 (m, 3H, Ar-H), 6.43 (s, 2H, Ar-H), 3.81 (s, 9H, OCH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.22, 159.38, 154.04, 153.96, 153.95, 147.13, 142.46, 135.17, 129.90, 127.20, 126.61, 124.82, 124.41, 117.78, 117.09, 104.66, 60.51, 56.54; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{21}H_{18}O_6$: 366.1103, Obsd 366.1098.

(E)-3-(3-(4-Chlorophenyl)acryloyl)-2H-chromen-2-one **(6c)**

Brown, R_f = 0.46, Yield: 70 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.40 (s, 1H, Ar-H), 7.92 (d, 1H), 7.61 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.58 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.43 (d, 1H), 7.38-7.28 (m, 3H, Ar-H), 7.25 (d, 2H, Ar-H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.18, 159.32, 153.41, 147.08, 142.48, 135.10, 133.91, 133.64, 129.82, 128.66, 128.54, 126.54, 124.80, 125.72, 117.76, 117.07; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{18}H_{11}ClO_3$: 310.0397, Obsd 310.0394.

(E)-3-(3-(4-Nitrophenyl)acryloyl)-2H-chromen-2-one **(6d)**

Brown, R_f = 0.43, Yield: 74 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.35 (s, 1H, Ar-H), 8.12 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.95 (d, 1H), 7.86 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.58 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.53-7.41 (m, 4H, Ar-H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.20, 159.26, 154.12, 147.43, 147.28, 142.43, 141.45, 133.84, 130.11, 128.56, 128.05, 126.52, 124.74, 123.29, 117.71, 116.91; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{18}H_{11}NO_5$: 321.0637, Obsd 321.0635.

3-Cinnamoyl-2H-chromen-2-one **(6e)**

Light orange, R_f = 0.45, Yield: 65 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.37 (s, 1H, Ar-H), 7.97 (d, 1H), 7.58 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.53 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.45 (d, 1H), 7.41 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.39-7.32 (m, 4H, Ar-H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.24, 159.25, 154.12, 147.46, 142.47, 135.97, 134.05, 129.24, 128.43, 128.21, 128.10, 126.91, 124.73, 123.56, 117.73, 116.98; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{18}H_{12}O_3$: 276.0786, Obsd 276.0789.

(E)-3-(3-(4-Hydroxyphenyl)acryloyl)-2H-chromen-2-one **(6f)**

Dark brown, R_f = 0.43, Yield: 75%. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.41 (s, 1H, Ar-H), 7.96 (d, 1H), 7.60 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.56 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.47 (d, 1H), 7.44-7.34 (m, 3H, Ar-H), 6.79 (d, 2H, *J* = 7.5 Hz, Ar-H), 4.12 (s, 1H, OH); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.24, 159.45, 157.46, 154.05, 146.92, 142.34, 135.14, 129.97, 128.25, 127.34, 126.78, 124.80, 124.48, 117.79, 117.12, 114.34; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{18}H_{12}O_4$: 292.0736, Obsd 292.0741.

(E)-3-(3-(2-Methoxyphenyl)acryloyl)-2H-chromen-2-one **(6g)**

Brown, R_f = 0.42, Yield: 69 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.43 (s, 1H, Ar-H), 8.01 (d, 1H), 7.64 (d, 1H, *J* = 7.8 Hz, Ar-H), 7.58-7.50 (m, 2H, Ar-H), 7.39-7.30 (m, 3H, Ar-H), 7.02 (m, 2H, Ar-H), 6.81 (d, 1H), 3.80 (s, 3H, OCH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.37, 159.48, 159.32, 154.08, 152.12, 146.95, 135.20, 134.68, 129.13, 128.31, 127.36, 125.46, 124.80, 124.66, 120.67, 117.84, 117.14, 114.29, 56.43; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{19}H_{14}O_4$: 306.0892, Obsd 306.0889.

General method for the preparation of Coumarin based Pyrazolines (7)

In a round bottom flask, a solution of coumarin chalcones (**6**, 0.5g) and hydrazine hydrate (1eq) in glacial acetic acid (5ml) was added and refluxed at 80^{o}C for 8 h. The progression of reaction was observed by TLC. After completion of reaction, mixture was poured into the crushed ice with continuous stirring and kept aside for 15-20 minutes. The precipitates obtained was filtered

and washed with water. The crude product was purified by column chromatography with 10-15% EtOAc in hexane to afford the desired product (**7a-g**).

3-(1-Acetyl-5-(4-methoxyphenyl)-4, 5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7a)**

Light brown powder, R_f = 0.35, Mp 150-155°C, Yield: 82.1 %, **^{1}H NMR** (400 MHz, $CDCl_3$:DMSO d6; 9:1) δ 8.46 (d, 1H, *J* = 5.2 Hz, Ar-H),7.68-7.61 (m, 2H, Ar-Hs), 7.37-7.34 (m, 2H, Ar-Hs), 7.16-7.12 (m, 2H, Ar-Hs), 6.85-6.82 (m, 2H, Ar-Hs), 5.54-5.49 (m, 1H, Pyrazoline-H), 3.96-3.87 (m, 1H, Pyrazoline-H), 3.77 (dist. s, 3H, -OCH_3), 3.40-3.32 (m, 1H, Pyrazoline-H), 2.40 (dist. s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$:DMSO d6; 9:1) δ 168.56, 158.94, 158.80, 153.87, 150.56, 141.12, 133.75, 132.83, 128.87, 126.72, 124.94, 119.43, 118.66, 116.36, 114.01, 59.66, 55.15, 44.13, 21.95; **HRMS** (micro TOF-QII, HRMS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{21}H_{18}N_2O_4$: 363.1345, Obsd 363.1354.

3-(1-Acetyl-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7b)**

Brown powder, R_f = 0.47, Mp 190-195°C, Yield: 85.5 %, **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.41 (s, 1H, Ar-H),7.61-7.55 (m, 2H, Ar-H), 7.35-7.30 (m, 2H, Ar-H), 6.40 (s, 2H, Ar-H), 5.50 (dd, 1H, *J*= 4.8 & 6.8 Hz), 3.88 (dist. dd, 1H, pyrazolines-H), 3.81 (s, 6H, OCH_3), 3.77 (s, 3H, OCH_3), 3.42 (dist. dd, 1H, Pyrazoline-H), 2.43 (s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.15, 159.27, 154.19, 153.66, 151.02, 141.14, 141.12, 137.31, 133.07, 128.90, 125.11, 119.68, 118.83, 116.78, 102.97, 102.42, 60.85, 60.67, 56.21, 44.46, 29.78, 22.12; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{23}H_{22}N_2O_6$: 423.1556, Obsd 423.1569.

3-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7c)**

Light green, R_f = 0.42, Mp 170-175°C, Yield: 78.1 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.43 (s, 1H, Ar-H),7.61-7.56 (m, 2H, Ar-H), 7.35-7.24 (m, 4H, Ar-H),7.14 (d, 2H, *J* = 6.8 Hz, Ar-H), 5.53 (dd, 1H, *J*= 4.8 & 7.2 Hz), 3.94 (dist. dd, 1H, pyrazolines-H), 3.37 (dist. dd, 1H, Pyrazoline-H), 2.40 (s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.05, 159.30, 154.22, 150.79, 141.17, 140.06, 133.56, 133.07, 129.13, 128.92, 127.14, 125.12, 119.57, 118.83, 116.79, 59.94, 44.27, 22.06; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{20}H_{15}ClN_2O_3$: 366.0771, Obsd 366.0767.

3-(1-Acetyl-5-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7d)**

Light brown, R_f = 0.39, Mp 135-140°C, Yield: 73.5 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.23 (s, 1H, Ar-H),7.92 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.42-7.37 (m, 2H, Ar-H), 7.33-7.08 (m, 4H, Ar-H), 5.38 (dd, 1H, *J*= 5.2 & 7.8 Hz), 3.76 (dist. dd, 1H, pyrazolines-H), 3.13 (dist. dd, 1H, Pyrazoline-H), 2.31 (s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.01, 159.28, 154.19, 150.52, 150.24, 144.76, 141.56, 134.34, 133.10, 128.98, 126.63, 125.05, 124.08, 118.48, 116.42, 59.72, 43.54, 21.82; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{20}H_{15}N_3O_5$: 377.1012, Obsd 377.1015.

3-(1-Acetyl-5-phenyl-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7e)**

Brown, R_f = 0.40, Mp 170-175°C, Yield: 66.1 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.30 (s, 1H, Ar-H),7.49-7.42 (m, 3H, Ar-H), 7.21-7.04 (m, 6H, Ar-H), 5.42 (dd, 1H, *J*= 4.6&7.2 Hz), 3.80 (dist. dd, 1H, pyrazolines-H), 3.22 (dist. dd, 1H, Pyrazoline-H), 2.02 (s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.08, 159.26, 154.21, 150.50, 150.27, 144.78, 141.56, 134.32, 133.13, 128.94, 126.61, 125.08, 124.11, 118.45, 116.43, 59.76, 43.99, 21.93; **HRMS** (microTOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{20}H_{16}N_2O_3$: 332.1161, Obsd 332.1156.

3-(1-Acetyl-5-(4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7f)**

Brown, R_f = 0.45, Mp 190-195°C, Yield: 65.0 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.19 (s, 1H, Ar-H),7.39-7.10 (m, 4H, Ar-H), 6.89 (d, 2H, *J* = 7.5 Hz, Ar-H), 6.70-6.58 (m, 2H, Ar-H), 5.25 (dd, 1H, *J*= 4.8 & 7.0 Hz), 3.64 (dist. dd, 1H, pyrazolines-H), 3.13 (dist. dd, 1H, Pyrazoline-H), 2.75 (s, 1H, OH), 2.33 (s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.14, 159.28, 154.25, 153.60, 151.07, 141.18, 141.14, 137.33, 133.11, 128.90, 125.11, 119.72, 118.86, 116.78, 103.04, 60.14, 44.51, 21.97; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{20}H_{16}N_2O_4$: 348.1110, Obsd 348.1114.

3-(1-Acetyl-5-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one **(7g)**

Brown, R_f = 0.37, Mp 150-155°C, Yield: 75.9 %. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.41 (s, 1H, Ar-H), 7.56 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.33-7.25 (m, 3H, Ar-H), 7.16 (d, 1H, *J* = 7.0 Hz, Ar-H), 6.89-6.80 (m, 3H, Ar-H), 5.47 (dd, 1H, *J*= 4.6 & 7.0 Hz), 3.92 (dist. dd, 1H, pyrazolines-H), 3.76

(s, 3H, OCH_3), 3.35 (dist. dd, 1H, Pyrazoline-H), 2.36 (s, 3H, CH_3); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.11, 159.25, 154.24, 153.42, 151.11, 137.24, 131.19, 128.89, 127.91, 125.64, 125.61, 119.98, 119.72, 118.65, 116.67, 116.40, 112.14, 60.34, 56.45, 44.59, 22.16; **HRMS** (micro TOF-QII, MS, ESI): *m/z* $[M+H]^+$ Calculated for $C_{21}H_{18}N_2O_4$: 362.1267, Obsd 362.1271.

Biological evaluation

In-vitro anti-inflammatory activity

***In- vitro* COX inhibition assay:** The effect of synthesized compounds (**7a-g**) on COX-1 and COX-2 were evaluated using COX (ovine) inhibitor screening assay EIA kit (Catalogue No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) according to manufacturer's instructions. The compounds were dissolved in dimethylsulfoxide (DMSO). The enzyme COX-1 and COX-2 (10 µL), heme (10 µL) and compounds (20 µL) were added to the supplied reaction buffer solution (950 µL, 0.1 M Tris-HCl, pH 8 containing 5 mM ethylenediaminetetraacetate and 2 mM phenol). The mixture of these solutions was incubated for a period of 10 min at 37 °C, and then COX reactions were initiated by adding arachidonic acid (10 µL, making final concentration 100 µM) solution. The COX reactions were quenched by addition of HCl (1 M, 50 µL) after 2 min and then saturated stannous chloride (100 µL) was added and again incubated for 5 min at room temperature. The $PGF_{2\alpha}$ formed by COX reactions was quantified by EIA. The pre-coated 96-well plate containing compounds was incubated for 18 h at room temperature. After incubation, the plate was washed to remove any unbound reagent and then Ellman's reagent (200 µL), was added followed by incubation for 60 min (until the absorbance of B_o well is in the range 0.3-1.0 A. U.) at room temperature. The plate was then read by an ELISA plate reader at 410 nm.

Inhibition of albumin denaturation: The anti-inflammatory activity of synthesized compounds **7a-g** was determined by using the procedure for inhibition of albumin protein denaturation as previously reported.[2]

Membrane stabilization: Membrane stabilization assays such as Heat-induced hemolysis and Hypotonicity-induced hemolysis was performed by following the already reported method.[2]

In-vivo **anti-inflammatory and analgesic activity:** The analgesic and anti-inflammatory activity of the compounds were performed by using male Wistar rat (4–6 weeks old), weighing 150-200 g procured from Central Animal House facility of ISF College of Pharmacy, Moga, Punjab India. They were housed in polypropylene cages with four of them in one cage and maintained at a temperature range of 22–24˚C with access to standard animal food and clean drinking water. Our animal house and breeding facility is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and CPCSEA guidelines were followed (CPCSEA approval number ISFCP/IAEC/CPCSEA/2017/354). The analgesic and anti-inflammatory activity of the compounds were carried out using our previously reported procedure.[3, 4]

Estimation of Cytokine levels: The cytokines level were estimated *in vivo* by using rat TNF-α, IL-6, and IL-1β immunoassay kit (KRISHGEN Biosystem, Ashley Ct, Whittier, CA).[4]

Acute toxicity studies: Acute toxicity studies were performed with synthesized compounds on either sex rats according to OECD guidelines.[3, 4] Animals were fasted for 4h prior to dosing and for further 2h after dosing. The first group was treated as control and the second group was treated with synthesized compound at a dose of 50 mg/kg. The third group was treated with synthesized compound at a dose of 300 mg/kg, and the fourth group was treated with synthesized compound at a dose of 2000 mg/kg. All treatments were given orally in a single dose. First 4h the animals were observed continuously followed by periodic monitoring for 24h. Thereafter, the animals were observed once or twice daily for a period of 14 days.

Molecular docking study: The molecular docking studies on the synthesized compounds were carried out by molecular docking software gold version 5.0 for predicting the binding affinity of synthesized compounds with COX-2, COX-1 and 5-LOX. The X-ray crystallographic structure of the selected proteins COX-2 (PDB 3LN1), COX-1 (PDB 3KK6) and 5-LOX (PDB 3V99) were procured from protein data bank.[5-8] The protein and ligand preparation were carried out using MOE (molecular Operating Environment). All the synthesized compounds were energy minimized by selecting force field MMFF94X with gradient value of 0.0001 Kcal/mol. The energy minimized compounds were docked in the active site of the target protein. Docking

protocol was validated by redocking of co-crystallized structure and RMSD value was calculated.

Table S1: Effect of coumarin-pyrazolines **7a-g** on albumin denaturation.

Treatment	Concentration (µg/ml)	Absorbance	% protection against denaturation
Control	-	0.683±0.005	-
7a	100	0.200±0.008***	70
	50	0.212±0.01***	68
	25	0.220±0.004***	67
	10	0.250±0.002***	63
7b	100	0.350±0.006**	48
	50	0.375±0.003*	45
	25	0.395±0.001*	42
	10	0.400±0.009*	41
7c	100	0.398±0.003**	44
	50	0.405±0.007**	40
	25	0.420±0.01*	38
	10	0.443±0.005	34
7d	100	0.280±0.007***	59
	50	0.300±0.005***	56
	25	0.315±0.001***	53
	10	0.330±0.002***	51
7e	100	0.315±0.004**	53
	50	0.330±0.007**	51
	25	0.365±0.01**	46
	10	0.370±0.002*	45
7f	100	0.350±0.004***	48
	50	0.366±0.008***	46
	25	0.386±0.006***	43
	10	0.400±0.002***	41
7g	100	0.390±0.007**	42
	50	0.383±0.002**	41
	25	0.415±0.006**	39
	10	0.425±0.001*	37
Etoricoxib	100	0.173±0.007***	74
	50	0.189±0.009***	72
	25	0.190 ±0.005***	72
	10	0.198±0.003***	71

Each value represents the mean ± SD. n=3, A significant difference between the test and standard group were considered when test group = *($p<0.05$), **($p<0.01$), *** ($p<0.001$) using Bonferroni test by applying two-way ANOVA.

Table S2: Effect of coumarin-pyrazolines on heat induced hemolysis

Treatment	Concentration	Absorbance	% protection against heat induced hemolysis
Control	-	0.46	-
7a	100	0.14±0.005***	69
	50	0.15±0.008***	67
	25	0.17±0.003***	63
	10	0.18±0.007***	60
7b	100	0.23±0.001**	50
	50	0.25±0.006*	45
	25	0.26±0.002*	43
	10	0.27±0.008*	41
7c	100	0.25±0.01**	45
	50	0.27±0.003*	41
	25	0.28±0.006*	39
	10	0.29±0.009*	36
7d	100	0.19±0.002***	58
	50	0.20±0.01***	56
	25	0.21±0.004***	54
	10	0.23±0.008***	50
7e	100	0.24±0.003**	47
	50	0.25±0.006**	45
	25	0.27±0.004**	41
	10	0.28±0.008*	39
7f	100	0.24±0.007***	47
	50	0.25±0.009***	45
	25	0.26±0.004***	43
	10	0.27±0.006***	41
7g	100	0.22±0.009**	51
	50	0.23±0.004**	50
	25	0.25±0.01**	45
	10	0.26±0.006*	43
Etoricoxib	100	0.11±0.003***	76
	50	0.10±0.007***	78
	25	0.12±0.005***	73
	10	0.16±0.006***	65

Each value represents the mean ± SD. n=3, A significant difference between the test and standard group were considered when test group = *($p<0.05$), **($p<0.01$), ***($p<0.001$) using Bonferroni test by applying two-way ANOVA.

Table S3: Effect of coumarin-pyrazolines on hypotonicity-induced hemolysis

Treatment	Concentration	Absorbance	% protection against hemolysis
Control	-	0.570±0.004	-
7a	100	0.180±0.008***	68
	50	0.190±0.001***	67
	25	0.210±0.005***	63
	10	0.215±0.007***	62
7b	100	0.290±0.001**	50
	50	0.300±0.005**	48
	25	0.320±0.007*	44
	10	0.330±0.004*	43
7c	100	0.260±0.008**	55
	50	0.270±0.006**	53
	25	0.290±0.002**	50
	10	0.280±0.009**	51
7d	100	0.240±0.003***	58
	50	0.260±0.004***	55
	25	0.268±0.005***	53
	10	0.275±0.01***	52
7e	100	0.300±0.003**	48
	50	0.320±0.007**	44
	25	0.330±0.008*	43
	10	0.350±0.004*	39
7f	100	0.290±0.006***	50
	50	0.300±0.008***	48
	25	0.310±0.004***	46
	10	0.323±0.003***	44
7g	100	0.270±0.01**	53
	50	0.290±0.004**	50
	25	0.300±0.002*	48
	10	0.330±0.007*	43
Etoricoxib	100	0.174±0.005***	69
	50	0.192±0.008***	66
	25	0.215±0.01***	62
	10	0.217±0.007***	52

Each value represents the mean ± SD. n=3, A significant difference between the test and standard group were considered when test group = *($p<0.05$), **($p<0.01$), ***($p<0.001$) using Bonferroni test by applying two-way ANOVA.

Table S4: Comparison of synthesized compounds binding ability to COX-1 and COX-2 enzyme

Compound	GOLD SCORE		
	COX-2	**COX-1**	**5-LOX**
7a	88.25	33.50	36.44
7b	82.05	35.77	42.01
7c	84.98	36.27	33.18
7d	59.90	36.50	35.14
7e	73.76	35.65	28.21
7f	79.74	34.87	31.72
7g	70.44	36.75	37.79
Celecoxib	96.42	36.28	-

Table S5: Virtual ADME (absorption, distribution, metabolism, excretion) and molecular property prediction of coumarin based pyrazolines **7a-g**.

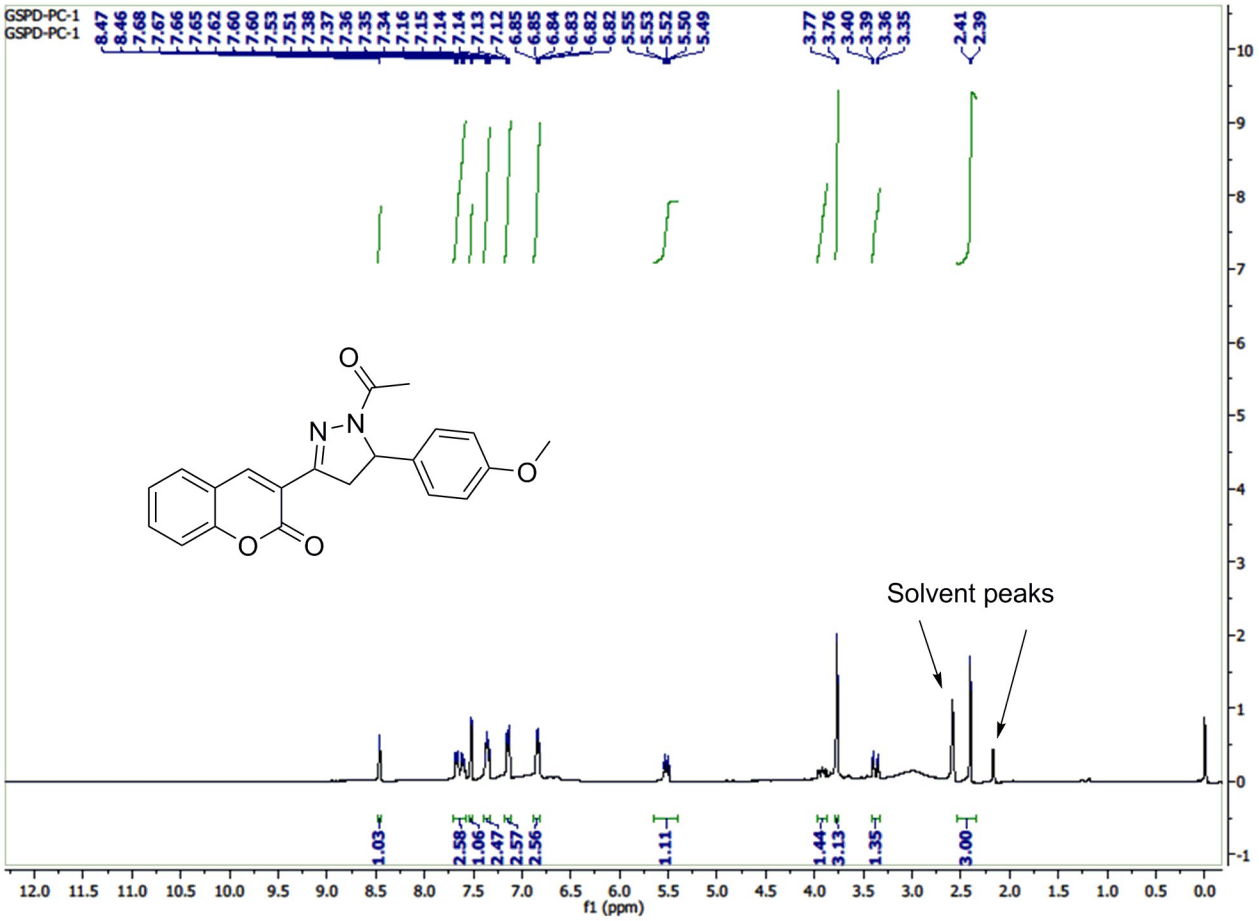
Compd	tPSA[a]	%Abs[b]	MW[c]	ROB [d]	HBD[e]	HBA[f]	MR[g]	ILogP[h]	LogS[i]
Rule	**≤140 Å²**	**-**	**≤1500**	**≤110**	**≤15**	**≤10**	**40-110**	**<5**	**>-4**
7a	72.11	84.12	362.38	4	0	5	109.56	2.47	-4.07
7b	69.59	84.99	408.45	6	0	6	120.82	2.01	-3.95
7c	41.90	94.54	352.81	3	0	3	106.35	3.48	-4.31
7d	87.72	78.73	363.37	4	0	5	110.16	2.03	-3.78
7e	41.90	94.54	318.37	3	0	3	101.34	2.99	-3.72
7f	62.13	87.56	334.37	3	1	4	103.36	2.43	-3.58
7g	72.11	84.12	362.38	4	0	5	109.56	2.47	-4.07

[a] Topological polar surface area; [b] Absorption; [c] Molecular weight; [d] number of rotatable bonds; [e] Number of hydrogen bond donors; [f] Number of hydrogen bonds acceptors; [g] Molar refractivity; [h] Logarithm of compound partition coefficient between *n*-octanol and water; [i] Logarithm of water solubility.

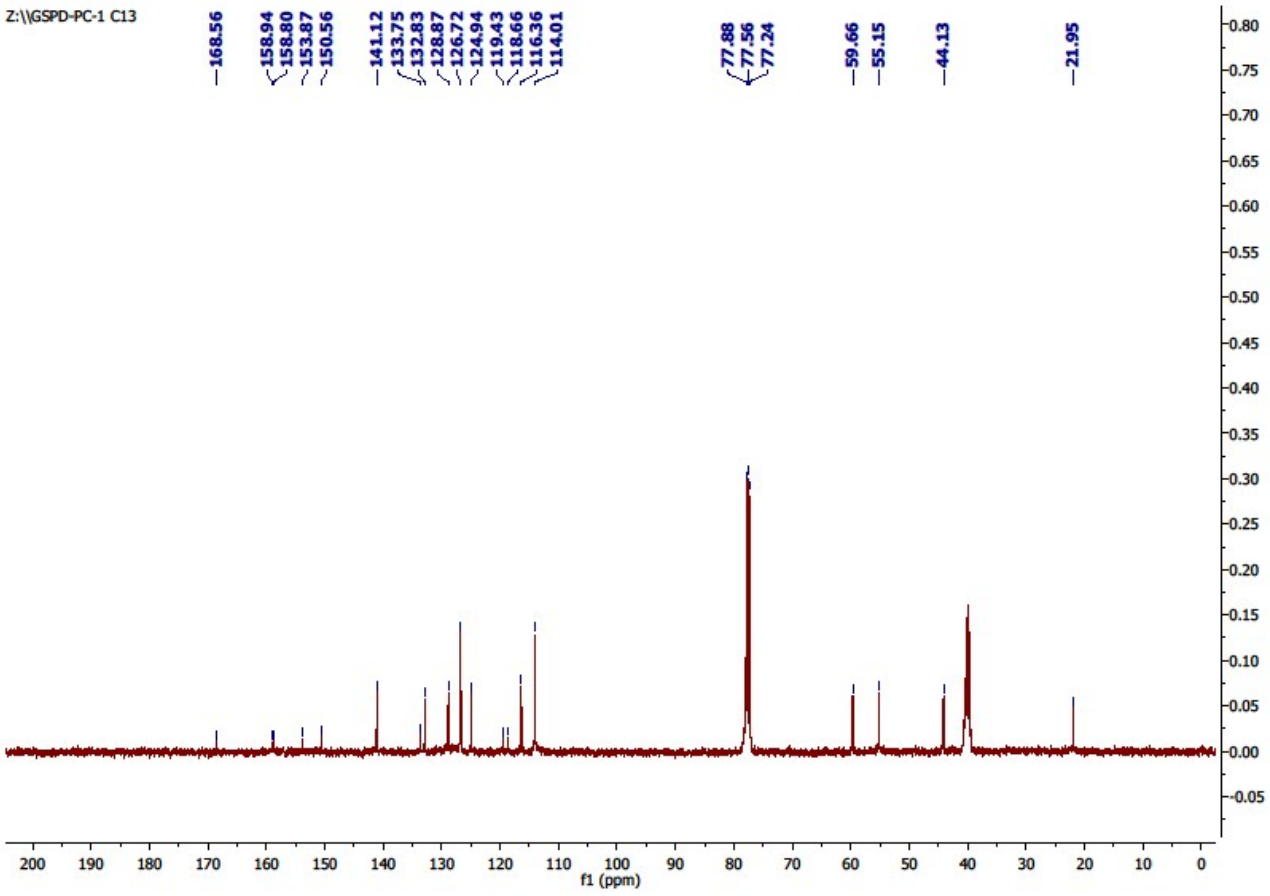
5-LOX Inhibition by Compound 7a: The procedure for screening of compound **7a** against 5-LOX inhibition was used as described in literature.[9]

Characterization data

^{1}H NMR of compound **7a**



^{13}C NMR of compound **7a**



HRMS of compound **7a**

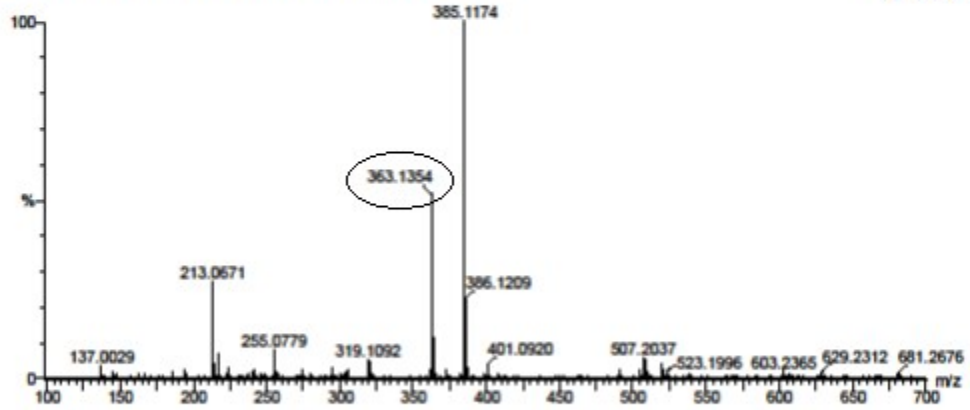
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
60 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 10-32 H: 10-35 N: 0-2 O: 0-8

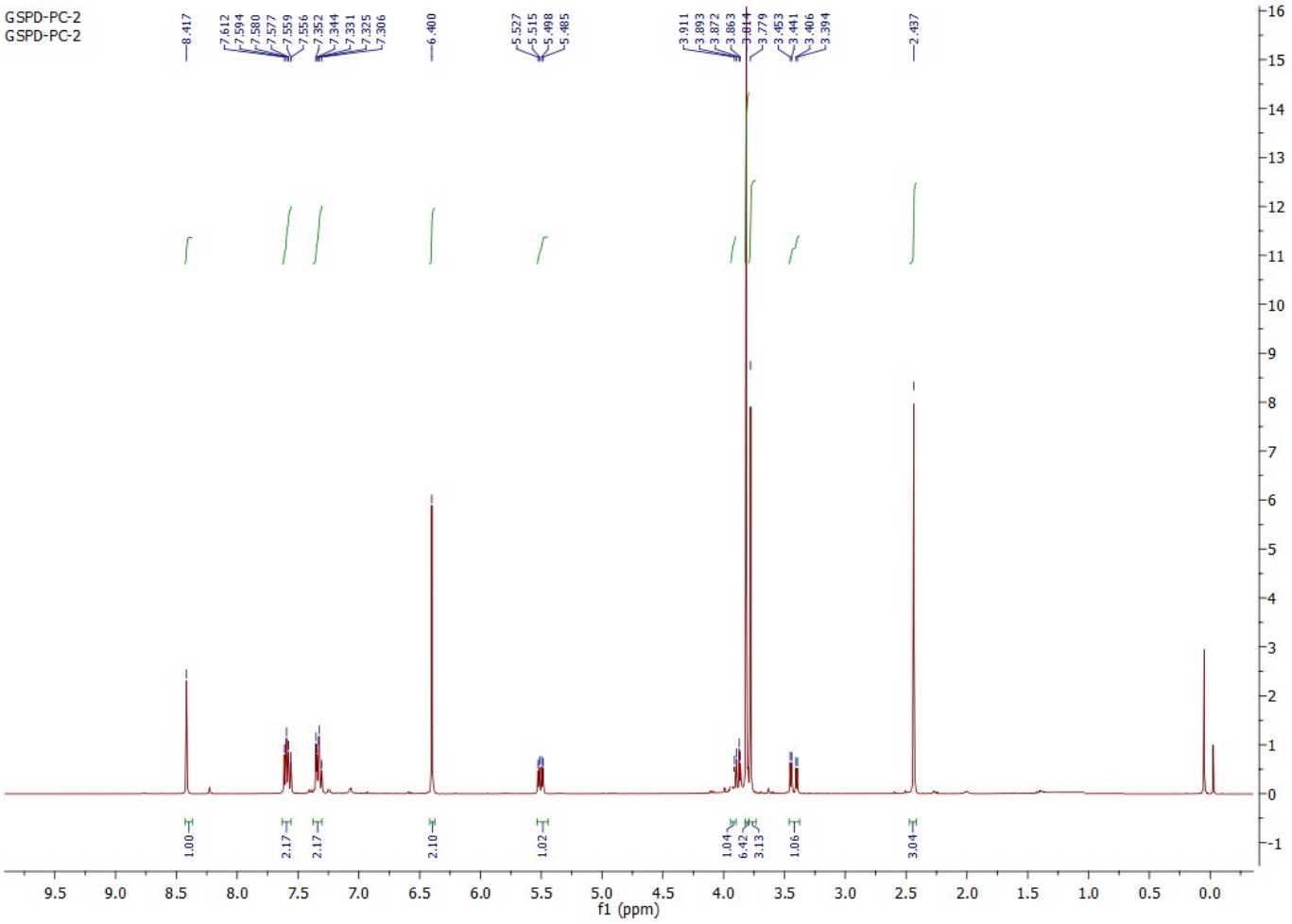
Sample Name : GSPD-PC-1
Test Name : HRMS-1
261118-GSPD-PC-1- 17 (0.174) AM2 (Ar,19000.0,0.00,0.00); Cm (17:20)

XEVO G2-XS QTOF
1: TOF MS ES+
2.55e+007

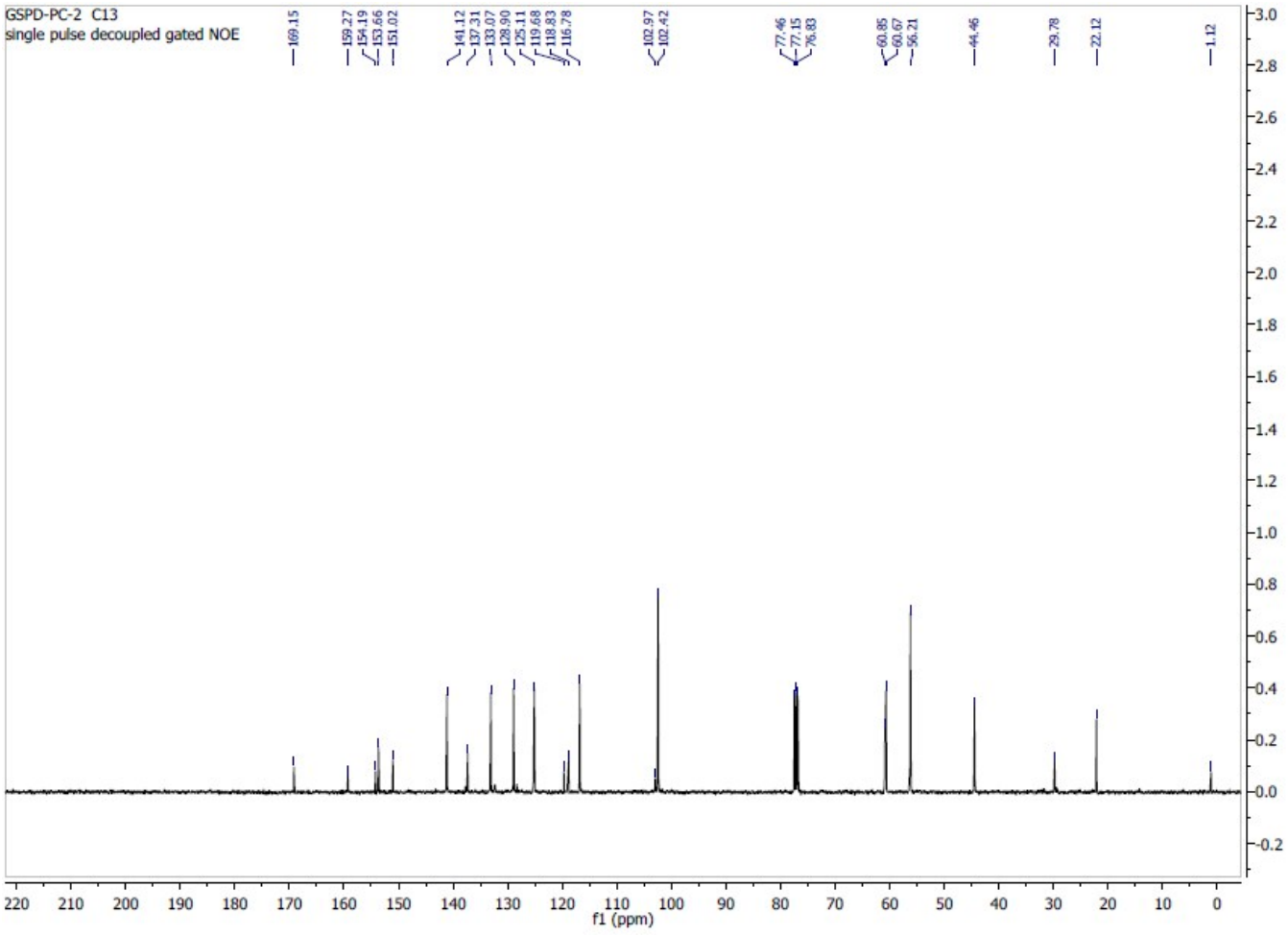


Minimum:				-1.5				
Maximum:		5.0	5.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
363.1354	363.1345	0.9	2.5	13.5	587.9	n/a	n/a	C21 H19 N2 O4

^{1}H NMR of compound **7b**



^{13}C NMR of compound **7b**



HRMS of compound **7b**

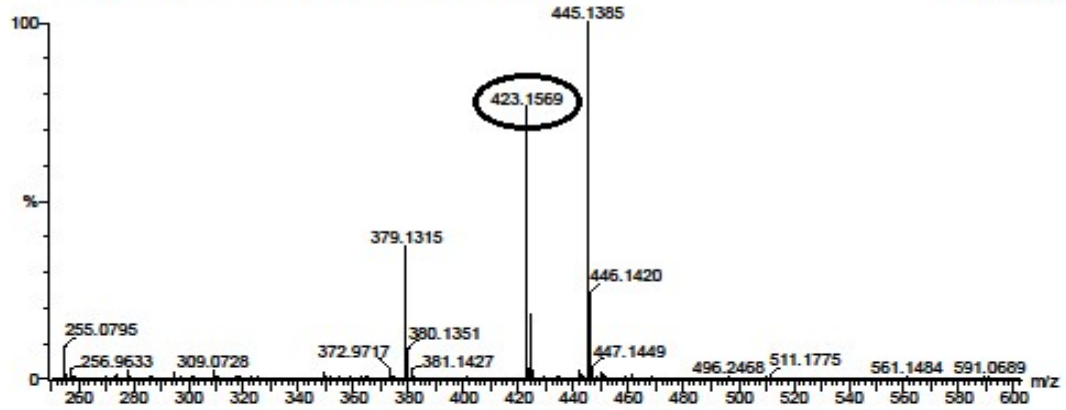
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 15.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
9 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 20-25 H: 21-25 N: 0-2 O: 0-6

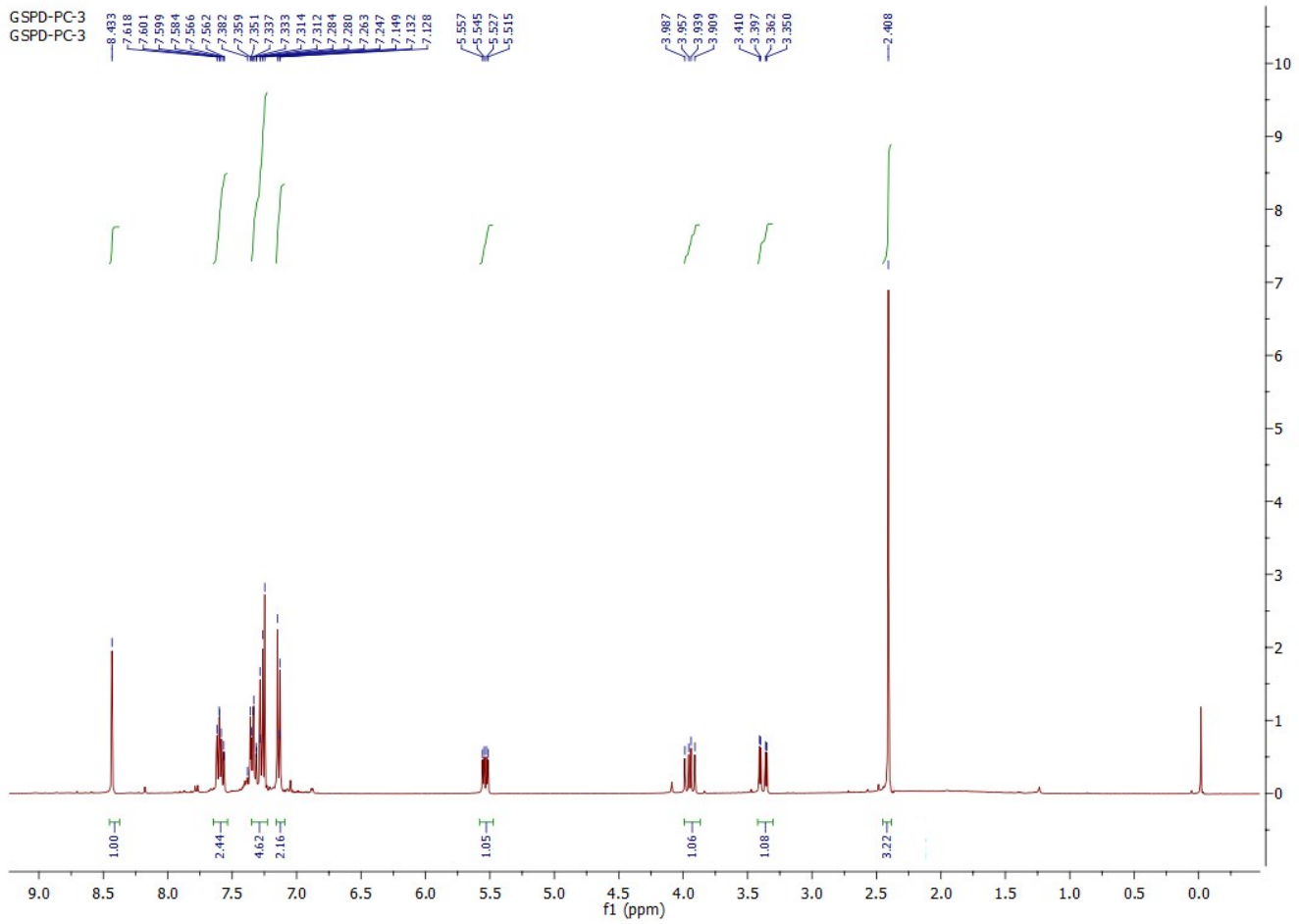
Sample Name : GSPD-PC-2
Test Name : HRMS-1
220518-GSPD-PC-2- 17 (0.174) AM2 (Ar,16000.0,0.00,0.00); Cm (17:19)

XEVO G2-XS QTOF
1: TOF MS ES+
1.39e+007

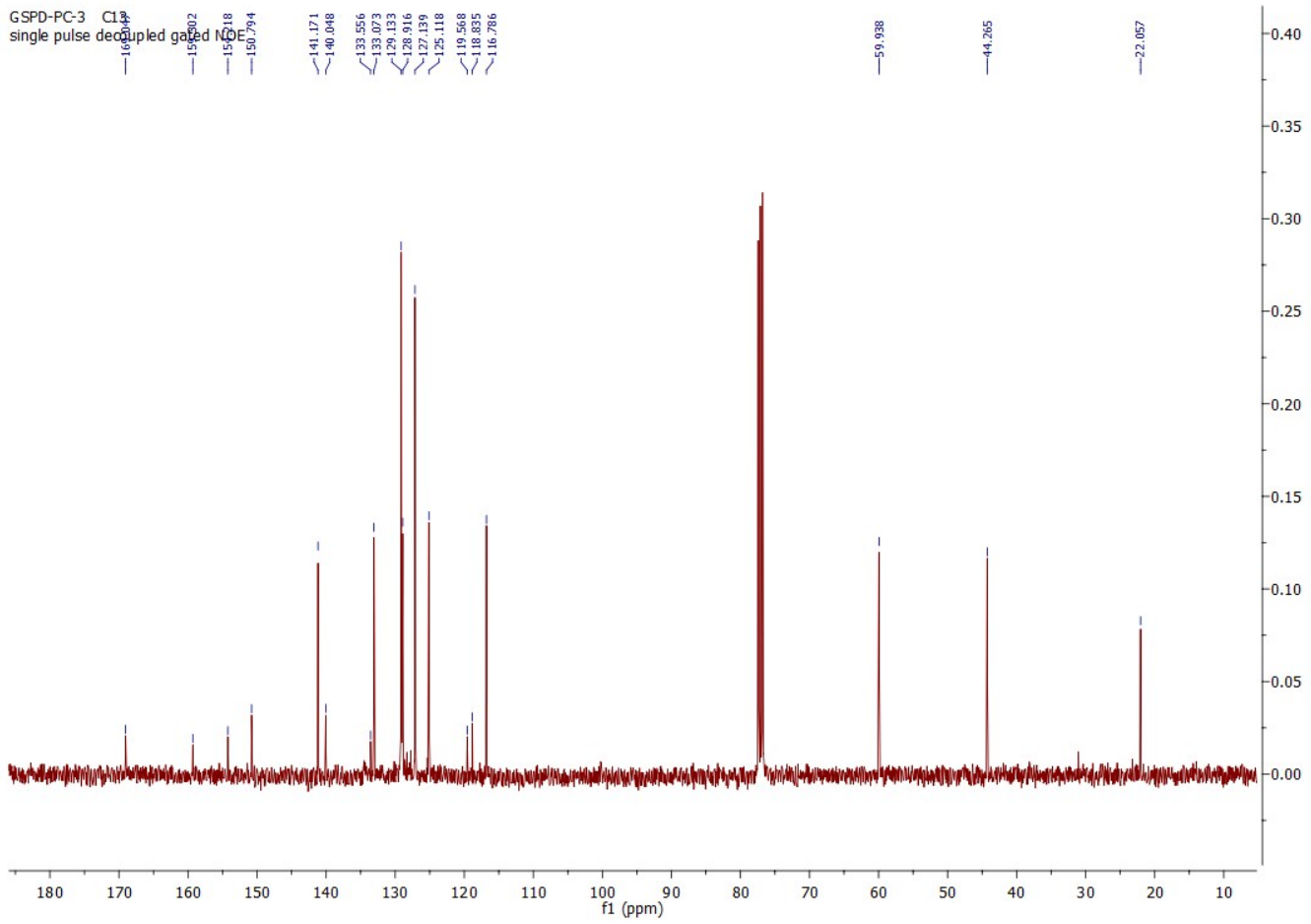


Minimum:				-1.5				
Maximum:		5.0	15.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
423.1569	423.1556	1.3	3.1	13.5	431.0	n/a	n/a	C23 H23 N2 O6

^{1}H NMR of compound **7c**



^{13}C NMR of compound **7c**



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