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Synthesis and Evaluation of Novel 5-{[2-Cyano-3-(substituted phenyl)prop-2-enoyl]amino}-2-hydroxy Benzoic Acid

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5-Aminosalicylic acid (5-ASA), also known as mesalamine is extensively used for the treatment of inflammatory conditions like ulcerative colitis (UC), inflammatory bowel disease (IBD) and colorectal cancer. Hence, the current work aimed at the synthesis of some novel derivatives of 5-ASA involving cyanoacetylation and subsequent Knoevenagel condensation reaction. The compounds were then characterized using physical and spectral data and screened for *in vitro* antioxidant and anti-inflammatory activities. The compounds showed promising *in vitro* antioxidant and anti-inflammatory activities among which compound **4e** (3,4-OCH_3) exhibited superior dual antioxidant activity in DPPH and nitric oxide assays greater than ascorbic acid and 5-ASA. Meanwhile, compound **4l** (-Cl) showed the strongest inhibition of protein denaturation and **4m** (-F) exhibited highest protection of HRBC membrane. Further, PASS analysis identified **4j** (4-$CH(CH_3)_2$), **4c** (3,4-OH) and **4m** (4-F) as promising anti-inflammatory candidates with favourable safety profiles when compared to 5-ASA. Overall, these derivatives showed good to moderate biological activity, suggesting for further evaluation under inflammatory conditions.

Keywords: 5-Aminosalicylic acid, Mesalamine, Cyanoacetylation, Knoevenagel condensation, Antioxidant, Anti-inflammatory.

INTRODUCTION

5-Aminosalicylic acid (5-ASA) is extensively used for the treatment of inflammatory bowel disease (IBD), ulcerative colitis (UC), Crohn's disease and colorectal cancer [1,2]. The pharmacological and toxicological studies have shown that 5-ASA exhibits a reduced anti-inflammatory effect at its target site as is largely absorbed in the upper gastrointestinal tract [3]. Hence, several derivatives of 5-ASA were developed by the formation of Schiff bases [4-7] and azo linkages [8-14]. These derivatives exhibit various pharmacological activities that include anti-inflammatory, antioxidant and anticarcinogenic activities but are still found to be associated with their own side effects [1,2]. Hence, there is a need to develop safer and more effective 5-ASA derivatives.

Literature data revealed that specific methods were not reported on the synthesis of 5-ASA derivatives by introducing nitrile group and active methylene moieties. Consequently, as a part of our research on cyanoacetylated derivatives [15-20], the present work aimed at synthesizing the 5-ASA derivatives in a two-step process, initially by cyanoacetylation of 5-ASA subsequently by the Knoevenagel condensation reaction [21,22] and to evaluate their *in vitro* anti-inflammatory and antioxidant activities. Further, *in silico* studies were planned to perform for assessing the physico-chemical properties, anti-inflammatory activity and toxicity of the compounds using online computational tools such as Molinspiration Cheminformatics, PASS analysis and Osiris Property Explorer, respectively.

EXPERIMENTAL

All the chemicals and solvents were procured from Sigma-Aldrich/HiMedia Labs/SR CHEM Chemicals. Melting points were measured using open capillaries on a Biotechnics India-BTI-34 electric melting point apparatus and are uncorrected. The purity of the synthesized compounds was verified on pre-coated alumina plates with silica gel and spots were visualized under a Biotechnics India-R/340/OB UV chamber. Infrared (IR) spectra recorded using the KBr pellet technique

on a Bruker OPUS_7.5.18 infrared spectrophotometer. ^{1}H NMR spectra were collected using Bruker® Avance spectrometers which operated at 500/400/300 MHz. ^{13}C NMR spectra were recorded at 125/100/75 MHz in DMSO, with tetramethylsilane (TMS) serving as the internal standard. Mass spectra obtained with Thermo Scientific Exactive Orbitrap LCMS, MaXis 10138 and Agilent SD/AD/INS/013 and 015 LCMS systems that feature ESI and APCI ionization sources.

General synthetic procedure for the preparation of novel 5-{[2-cyano-3-(substituted phenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid

To a solution of 5-ASA (**1**, 20 mM**)** in toluene (20 mL), 1-cyanoacetyl-3,5-dimethylpyrazole (20 mM**)** was added and refluxed at 110 ºC for 60 min to obtain 5-[(cyanoacetyl)-amino]-2-hydroxybenzoic acid (**2**). The resulting precipitate was separated by vacuum filtration, dried and recrystallized from rectified spirit [17-22]. In a second step, a mixture of compound **2** (10 mM), piperidine (0.35 mL) and acetic acid (1.35 mL) in toluene (50 mL), various substituted benzaldehydes (**3a-o**, 10 mM) were added. The mixture was then refluxed at 110 ºC along with continuous stirring for about 5-6 h to obtain the title compounds **4a-o**. Reactions were monitored with TLC and products were recrystallized from rectified spirit (**Scheme-I**). A total of 15 compounds were synthesized using the above-described procedure and characterized based on their physical and spectral data including IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic analyses.

5-{[2-Cyano-3-phenylprop-2-enoyl]amino}-2-hydroxybenzoic acid (4a): Yield: 55.8%; m.p.: 270-272 ºC; R_f: 0.76; IR (KBr, ν_{max}, cm^{-1}): 3450 (OH *str.*), 3347 (NH *str.*), 2217 (C≡N *str.*), 1686 (C=O *str.*), 1620 (C=O *str.*, amide I), 1560 (NH bend, amide II); ^{1}H NMR (DMSO, 400 MHz) δ ppm: 6.99-7.01 (dd, 1H, Ar), 7.61-7.62 (d, 3H, Ar), 7.80-7.83 (dd, 1H, Ar), 8.00-8.01 (d, 2H, Ar), 8.17-8.18 (d, 1H, Ar), 8.30 (s, 1H, C=CH), 10.41 (s, 1H, NH); ^{13}C NMR (DMSO, 125 MHz) δ ppm: 107.64, 113.12, 116.60, 117.70, 122.74, 129.22, 129.73 (2C), 130.28, 130.51 (2C), 132.39, 132.89, 151.17, 158.34, 160.71, 172.02; Mass (m.w. 308) *m/z*: 309 (M+H)$^+$.

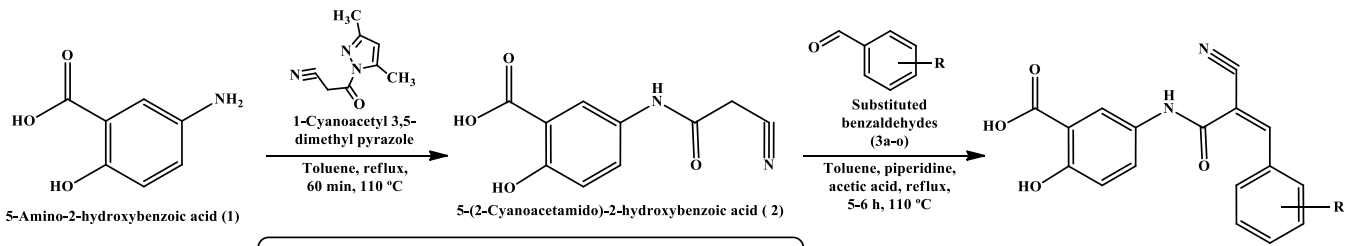
5-{[2-Cyano-3-(4-hydroxyphenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4b): Yield: 68.10%; m.p.: 247-249 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3441 (OH, NH *str.*), 2215 (C≡N *str.*), 1670 (C=O *str.*), 1640 (C=O *str.*, amide I), 1553 (NH bend, amide II); ^{1}H NMR (DMSO, 500 MHz) δ ppm: 6.86-6.90 (dd, 1H, Ar), 6.96-6.97 (dd, 2H, Ar), 7.71-7.74 (dd, 1H, Ar), 7.91-7.93 (dd, 2H, Ar), 8.093 (d, 1H, Ar), 8.098 (s, 1H, C=CH), 10.16 (s, 1H, NH); ^{13}C NMR (DMSO, 75 MHz) δ ppm: 102.10, 114.53, 116.25 (2C), 116.68, 117.01, 122.43, 122.91, 127.78, 129.30, 132.90 (2C), 150.37, 158.17, 160.76, 161.83, 171.51; Mass (m.w. 324) *m/z*: 325 (M+H)$^+$.

5-{[2-Cyano-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-amino}-2-hydroxybenzoic acid (4c): Yield: 93.30%; m.p.: 252-254 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3443 (OH *str.*), 3220 (NH *str.*), 2217 (C≡N *str.*), 1671 (C=O *str.*), 1610 (C=O *str.*, amide I), 1559 (NH bend, amide II); ^{1}H NMR (DMSO, 500 MHz) δ ppm: 6.90-6.97 (dd, 2H, Ar), 7.33-7.35 (dd, 1H, Ar), 7.59-7.64 (d, 1H, Ar), 7.75-7.78 (dd, 1H, Ar), 8.04 (s, 1H, C=CH), 8.12-8.13 (d, 1H, Ar), 9.70 (s, 1H, NH), 10.17 (s, 2H, OH); ^{13}C NMR (DMSO, 100 MHz) δ ppm: 101.78, 115.54, 116.06, 116.32, 116.51, 117.11, 122.68, 123.37, 125.25, 127.40, 129.22, 145.81, 150.62, 150.93, 158.25, 160.94, 171.95; Mass (m.w. 340) *m/z*: 341 (M+H)$^+$.

5-{[2-Cyano-3-(4-methoxyphenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4d): Yield: 98.80%; m.p.: 210-212 ºC; R_f: 0.55; IR (KBr, ν_{max}, cm^{-1}): 3440 (OH *str.*), 3331 (NH *str.*), 2214 (C≡N *str.*), 1677 (C=O *str.*), 1607 (C=O *str.*, amide I), 1555 (NH bend, amide II), 1267 (C-O-C *str.* asym), 1023 (C-O-C *str.* sym); ^{1}H NMR (DMSO, 400 MHz) δ ppm: 3.88 (s, 3H, OCH_3), 6.96-6.99 (d, 1H, Ar), 7.16-7.18 (d, 2H, Ar), 7.78-7.81 (dd, 1H, Ar), 8.01-8.03 (d, 2H, Ar), 8.15 (d, 1H, Ar), 8.21 (s, 1H, C=CH), 10.27 (s, 1H, NH); ^{13}C NMR (DMSO, 125 MHz) δ ppm: 56.12, 103.94, 113.22, 115.36 (2C), 117.20, 117.61, 122.74, 124.88,129.19, 130.37, 133.01 (2C), 150.72, 158.27, 161.15, 163.20, 172.01; Mass (m.w. 338) *m/z*: 339 (M+H)$^+$.

5-{[2-Cyano-3-(3,4-dimethoxyphenyl)prop-2-enoyl]-amino}-2-hydroxybenzoic acid (4e): Yield: 69.02%; m.p.: 261-263 ºC; R_f: 0.50; IR (KBr, ν_{max}, cm^{-1}): 3400 (OH *str.*), 3316 (NH *str.*), 2214 (C≡N *str.*), 1666 (C=O *str.*), 1614 (C=O *str.*, amide I), 1557 (NH bend, amide II), 1251 (C-O-C *str.* asym), 1026 (C-O-C *str.* sym); ^{1}H NMR (DMSO, 400 MHz)) δ ppm: 3.83, 3.88 (2s, 6H, OCH_3), 6.96-6.98 (d, 1H, Ar), 7.19-7.21 (d, 1H, Ar), 7.63-7.66 (dd, 1H, Ar), 7.71-7.72 (s, 1H, Ar), 7.77-7.80 (dd, 1H, Ar), 8.14 (d, 1H, Ar), 8.20 (s, 1H, C=CH), 10.27 (s, 1H, NH); ^{13}C NMR (DMSO, 100 MHz) δ ppm: 55.57 (2C), 101.99, 112.73, 113.31, 116.00, 117.16 (2C), 122.23, 123.24, 126.07, 128.73, 130.00, 147.79, 150.91, 151.62, 157.77, 160.91, 171.65; Mass (m.w. 368) *m/z*: 367 (M-H)$^-$.

5-{[2-Cyano-3-(3,4,5-trimethoxyphenyl)prop-2-enoyl]-amino}-2-hydroxybenzoic acid (4f): Yield: 88.44%; m.p.:



Code	R	Code	R	Code	R
4a	H	4f	3,4,5-OCH_3	4k	4-$N(CH_3)_2$
4b	4-OH	4g	3-OCH_3, 4-OH	4l	4-Cl
4c	3, 4-OH	4h	3,5-OCH_3, 4-OH	4m	4-F
4d	4-OCH_3	4i	4-CH_3	4n	4-Br
4e	3,4-OCH_3	4j	4-$CH(CH_3)_2$	4o	4-NO_2

Scheme-I: Synthesis of 5-{[2-cyano-3-(substituted phenyl)prop-2-enoyl]amino}-2-hydroxy benzoic acids (**4a-o**)

233-235 ºC; R_f: 0.60; IR (KBr, ν_{max}, cm^{-1}): 3405 (OH *str.*), 3331 (NH *str.*), 2214 (C≡N *str.*), 1673 (C=O *str.*), 1640 (C=O *str.*, amide I), 1553 (NH bend, amide II), 1254 (C-O-C *str.* asym), 990 (C-O-C *str.* sym); ^{1}H NMR (DMSO, 500 MHz) δ ppm: 3.78 (s, 3H, OCH_3), 3.84 (s, 6H, $(OCH_3)_2$), 6.96-6.98 (d, 1H, Ar), 7.41 (s, 2H, Ar), 7.78-7.80 (dd, 1H, Ar), 8.13-8.14 (d, 1H, Ar), 8.21 (s, 1H, C=CH), 10.33 (s, 1H, NH); ^{13}C NMR (DMSO, 125 MHz) δ ppm: 56.48 (2C), 60.78, 105.99, 108.39 (2C), 113.29, 117.06, 117.67, 122.71, 127.54, 129.12, 130.25, 141.60, 151.26 (2C), 153.39, 158.34, 160.83, 172.04; Mass (m.w. 398) *m/z*: 399 $(M+H)^+$.

5-{[2-Cyano-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4g): Yield: 77.96%; m.p.: 267-269 ºC; R_f: 0.58; IR (KBr, ν_{max}, cm^{-1}): 3450 (OH *str.*), 3330 (NH *str.*), 2210 (C≡N *str.*), 1679 (C=O *str.*), 1610 (C=O *str.*, amide I), 1535 (NH bend, amide II), 1241 (C-O-C *str.* asym), 1017 (C-O-C *str.* sym); ^{1}H NMR (DMSO, 300 MHz) δ ppm: 3.82 (s, 3H, OCH_3), 6.93-6.97 (dd, 2H, Ar), 7.50-7.53 (d, 1H, Ar), 7.69 (s, 1H, Ar), 7.75-7.78 (dd, 1H, Ar), 8.12 (d, 1H, Ar), 8.13 (s, 1H, C=CH), 10.19 (s, 1H, NH), 10.31 (s, 1H, OH); ^{13}C NMR (DMSO, 75 MHz) δ ppm: 55.58, 102.00, 112.70, 113.34, 115.99, 117.14 (2C), 122.22, 123.23, 126.04, 128.73, 129.98, 147.78, 150.89, 151.60, 157.74, 160.89, 171.60; M ass (m.w. 354) *m/z*: 355 $(M+H)^+$.

5-{[(2-Cyano-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4h): Yield: 93.75%; m.p.: 248-250 ºC; R_f: 0.66; IR (KBr, ν_{max}, cm^{-1}): 3441 (OH *str.*), 3310 (NH *str.*), 2211 (C≡N *str.*), 1664 (C=O *str.*), 1612 (C=O *str.*, amide I), 1550 (NH bend, amide II), 1246 (C-O-C *str.* asym), 1110 (C-O-C *str.* sym); ^{1}H NMR (DMSO, 400 MHz) δ ppm: 3.84 (s, 6H, $(OCH_3)_2$), 6.96-6.99 (d, 1H, Ar), 7.43 (s, 2H, Ar), 7.78-7.81 (dd, 1H, Ar), 8.14-8.15 (d, 1H, Ar), 8.16 (s, 1H, C=CH), 9.76 (s, 1H, NH), 10.23 (s, 1H, OH); ^{13}C NMR (DMSO, 75 MHz) δ ppm: 56.04 (2C), 102.28, 108.51 (2C), 112.58, 117.19 (2C), 121.94, 122.20, 128.78, 130.01, 140.79, 147.90 (2C), 151.22, 157.73, 160.84, 171.60; Mass (m.w. 384) *m/z*: 385 $(M+H)^+$.

5-{[2-Cyano-3-(4-methylphenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4i): Yield: 69.56%; m.p.: 256-258 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3453 (OH *str.*), 3290 (NH *str.*), 2210 (C≡N *str.*), 1666 (C=O *str.*), 1610 (C=O *str.*, amide I), 1548 (NH bend, amide II); ^{1}H NMR (DMSO, 300 MHz) δ ppm: 2.49 (s, 3H, CH_3), 6.95-6.98 (d, 1H, Ar), 7.38-7.41 (d, 2H, Ar), 7.75-7.79 (dd, 1H, Ar), 7.87-7.90 (d, 2H, Ar), 8.13-8.14 (d, 1H, Ar), 8.21 (s, 1H, C=CH), 10.32 (s, 1H, NH); ^{13}C NMR (DMSO, 75 MHz) δ ppm: 26.50, 107.73, 112.86, 115.95, 117.22, 122.25, 128.60, 129.44 (2C), 129.69, 130.78, 131.70 (2C), 137.02, 149.34, 157.92, 160.057, 171.57; Mass (m.w. 322) *m/z*: 323 $(M+H)^+$.

5-({2-Cyano-3-[4-(propan-2-yl)phenyl]prop-2-enoyl}-amino)-2-hydroxybenzoic acid (4j): Yield: 55.55%; m.p.: 239-241 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3450 (OH *str.*), 3343 (NH *str.*), 2216 (C≡N *str.*), 1677 (C=O *str.*), 1605 (C=O *str.*, amide I), 1557 (NH bend, amide II); ^{1}H NMR (DMSO, 300 MHz) δ ppm: 1.21-1.23 (d, 6H, $(CH_3)_2$), 2.92-3.01 (septet, 1H, CH), 6.95-6.98 (d, 1H, Ar), 7.45-7.48 (d, 2H, Ar), 7.76-7.80 (dd, 1H, Ar), 7.90-7.93 (d, 2H, Ar), 8.13-8.14 (d, 1H, Ar), 8.23 (s, 1H, C=CH), 10.33 (s, 1H, NH); ^{13}C NMR (DMSO, 75 MHz) δ ppm: 23.41 (2C), 33.56, 105.85, 112.56, 116.35, 117.21, 122.24, 127.30 (2C), 128.79, 129.57, 129.84, 130.34 (2C), 150.63, 153.65, 157.80, 160.38, 171.55; Mass (m.w. 350) *m/z*: 351 $(M+H)^+$.

5-({2-Cyano-3-[4-(N,N′-dimethylamino)phenyl]prop-2-enoyl}amino)-2-hydroxybenzoic acid (4k): Yield: 87.40%; m.p.: 232-234 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3451 (OH *str.*), 3333 (NH *str.*), 2207 (C≡N *str.*), 1667 (C=O *str.*), 1610 (C=O *str.*, amide I), 1550 (NH bend, amide II), 1373 (C-N *str. tert.*-amine); ^{1}H NMR (DMSO, 300 MHz) δ ppm: 3.03-3.05 (s, 6H, $(CH_3)_2$), 6.81-6.84 (d, 2H, Ar), 6.91-6.94 (d, 1H, Ar), 7.74-7.78 (dd, 1H, Ar), 7.88-7.91 (d, 2H, Ar), 8.06 (s, 1H, C=CH), 8.11-8.12 (d, 1H, Ar), 10.03 (s, 1H, NH); ^{13}C NMR (DMSO, 100 MHz) δ ppm: 39.56, 39.82, 97.57, 111.66 (2C), 112.76, 117.01, 117.97, 118.66, 122.17, 128.68, 130.2, 132.83 (2C), 150.54, 153.04, 157.62, 161.51, 171.68; Mass (m.w. 351) *m/z*: 352 $(M+H)^+$.

5-{[3-(4-Chlorophenyl)-2-cyanoprop-2-enoyl]amino}-2-hydroxybenzoic acid (4l): Yield: 80.00%; m.p.: 226-228 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3442 (OH *str.*), 3276 (NH *str.*), 2218 (C≡N *str.*), 1670 (C=O *str.*), 1647 (C=O *str.*, amide I), 1553 (NH bend, amide II) 1093 (C-Cl *str.*, Ar); ^{1}H NMR (DMSO, 500 MHz) δ ppm: 6.96-6.98 (dd, 1H, Ar),7.60-7.61 (d, 1H, Ar), 7.68-7.69 (d, 1H, Ar), 7.76-7.79 (dd, 1H, Ar), 7.98-8.00 (d, 2H, Ar), 8.13-8.14 (d, 1H, Ar), 8.27 (s, 1H, C=CH), 10.38 (s, 1H, NH); ^{13}C NMR (DMSO, 100 MHz) δ ppm: 107.73, 112.87, 115.94, 117.22, 122.20, 128.56, 129.45(2C), 129.66, 130.78, 131.70 (2C), 136.99, 149.33, 157.91, 160.04, 171.52; Mass (m.w. 343) *m/z*: 343, 345 (M, $M+2H)^+$.

5-{[2-Cyano-3-(4-fluorophenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4m): Yield: 60.12%; m.p.: 260-262 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3423 (OH *str.*), 3270 (NH *str.*), 2219 (C≡N *str.*), 1660 (C=O *str.*), 1644 (C=O *str.*, amide I), 1553 (NH bend, amide II)1198 (C-F *str.*, Ar); ^{1}H NMR (DMSO, 400 MHz) δ ppm: 6.98-7.00 (d, 1H, Ar), 7.45-7.50 (t, 2H, Ar), 7.79-7.82 (dd, 1H, Ar), 8.07-8.10 (dd, 2H, Ar), 8.16 (d, 1H, Ar), 8.29 (s, 1H, C=CH), 10.39 (s, 1H, NH); ^{13}C NMR (DMSO, 100 MHz) δ ppm: 107.26, 113.16, 116.58, 116.93, 117.15, 117.74, 122.72, 129.06, 129.21, 130.27, 133.23, 133.32, 150.00, 158.34, 160.67, 163.50, 172.01; Mass (m.w. 326) *m/z*: 325 $(M-H)^-$.

5-{[2-Cyano-3-(4-bromophenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4n): Yield: 86.95%; m.p.: 252-254 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3452 (OH *str.*), 3270 (NH *str.*), 2216 (C≡N *str.*), 1660 (C=O *str.*), 1644 (C=O *str.*, amide I), 1549 (NH bend, amide II) 1082, 951 (C-Br *str.*, Ar); ^{1}H NMR (DMSO, 500 MHz) δ ppm: 6.81-6.88 (dd, 2H, Ar), 6.90 (d, 1H, Ar), 7.71-7.73 (dd, 1H, Ar), 7.78-7.79 (dd, 2H, Ar), 8.06 (s, 1H, C=CH), 8.09-8.10 (d, 1H, Ar), 9.98 (s, 1H, NH); ^{13}C NMR (DMSO, 100 MHz) δ ppm: 108.69, 112.81, 115.75, 117.26, 122.20, 122.27, 128.59, 128.69, 129.64, 131.37, 132.31, 134.19, 134.79, 149.03, 157.92, 159.84, 171.53; Mass (m.w. 387) *m/z*: 385 $(M-2H)^-$, 387 (M).

5-{[2-Cyano-3-(4-nitrophenyl)prop-2-enoyl]amino}-2-hydroxybenzoic acid (4o): Yield: 66.85%; m.p.: 262-264 ºC; R_f: 0.57; IR (KBr, ν_{max}, cm^{-1}): 3443 (OH, NH *str.*), 2220 (C≡N *str.*), 1660 (C=O *str.*), 1644 (C=O *str.*, amide I), 1522 (NH bend, amide II), 1432 (NO_2 *str.*, asym), 1345 (NO_2 *str.*,

sym); ^{1}H NMR (DMSO, 500 MHz) δ ppm: 6.98-7.00 (d, 1H, Ar), 7.78-7.80 (dd, 1H, Ar), 8.15-8.18 (dd, 3H, Ar), 8.41 (s, 1H, C=CH), 8.42 -8.43 (d, 2H, Ar)**,** 10.51 (s, 1H, NH) ppm; ^{13}C NMR (DMSO, 75 MHz) δ ppm: 97.58, 111.85 (2C), 112.72, 117.00, 117.94, 118.66, 122.16, 128.68, 130.17, 132.81 (2C), 150.53, 153.04, 157.61, 161.50, 171.64; Mass (m.w. 353) *m/z*: 354 $(M+H)^+$.

***In vitro* antioxidant activity**

DPPH radical scavenging method: Solutions of the test compounds (100 μM) were mixed with 100 μM DPPH prepared in 95% ethanol. The subsequent procedure was conducted in accordance with the established protocol, utilizing ascorbic acid and 5-ASA as reference standards [23]. The percentage of scavenging activity was calculated using the following equation:

$$\text{Reduction of DPPH (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

NO radical scavenging method: Sodium nitroprusside (5 mM) prepared in phosphate-buffered saline (pH 7.4) was incubated with the test compound (100 μM) dissolved in 95% ethanol. Further procedure was carried out by following the standard procedure [23]. Ascorbic acid and 5-ASA served as reference standards. The percentage of NO radical scavenging activity was calculated using the following equation:

$$\text{NO free radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

***In vitro* anti-inflammatory activity**

Protein denaturation method: Samples were prepared by mixing 2 mL of each test compound solution (100 μM) by following the reported procedure [24]. 5-ASA served as the reference standard and the percentage of inhibition of denaturation was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

HRBC membrane stabilization assay: For the assay, mixtures containing 0.5 mL of test compound solution (100 μM) were prepared by following the reported procedure [25]. 5-ASA was used as a reference standard and the percentage of HRBC membrane stabilization was calculated using the following formula:

$$\text{Protection (\%)} = 100 - \left(\frac{\text{Optical density}_{\text{test}}}{\text{Optical density}_{\text{control}}} \right) \times 100$$

***In silico* studies**

Molinspiration Cheminformatics: Drug-likeness of the synthesized compounds (**4a-o**) and 5-ASA was evaluated using the Molinspiration Cheminformatics toolkit based on the Lipinski's rule of five, calculating key molecular properties (TPSA, molecular weight, log P, H-bond donors/acceptors, rotatable bonds and molecular volume) to predict their ADME profiles [26,27].

PASS analysis: Prediction of activity spectra for substances (PASS) is a computational tool that predicts multiple biological activities of organic, drug-like molecules based on their chemical structure, aiding in the evaluation of virtual compounds prior to synthesis and testing [28,29].

OSIRIS property explorer: OSIRIS is a computational tool used to predict and evaluate the properties and behaviour of organic compounds. It is commonly applied in drug discovery and development to assess toxicity, drug-likeness and other critical chemical properties [30,31].

RESULTS AND DISCUSSION

A series of novel 5-aminosalicylic acid (5-ASA) derivatives (**4a-o**) were synthesized through cyanoacetylation of 5-ASA followed by Knoevenagel condensation which involved coupling the active methylene group of 5-(2-cyanoacetamido)-2-hydroxybenzoic acid (**2**) with the carbonyl group of various substituted aromatic benzaldehydes. The condensation reaction was executed in toluene at 110 ºC in the presence of catalytic amounts of acetic acid and piperidine. The progress of the reaction was observed by thin-layer chromatography (TLC) and the crude products were purified by recrystallization using rectified spirit. The yields of the synthesized compounds ranged from 55.80% to 98.80%. Melting points were observed in the range of 210-270 ºC and R_f values in the range of 0.50 to 0.76. The structures of the synthesized compounds were characterized by spectroscopic techniques including IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry.

IR spectral analysis of the synthesized compounds **4a-o** revealed the characteristic absorption bands indicating the presence of specific functional groups. A distinct band observed in the range of 2220-2207 cm^{-1} was assigned to the stretching vibration of the nitrile group in all the synthesized compounds [32]. Moreover, broad absorption bands appearing between 3453 and 3316 cm^{-1} correspond to overlying N–H and O–H stretching vibrations. The C=O stretching bands were consistently observed at 1686-1660 cm^{-1} for the carboxylic acid group and at 1647-1607 cm^{-1} for the secondary amide functionality. Compounds **4d–4h** displayed additional characteristic bands at 1267-1241 cm^{-1} and 1026-990 cm^{-1} associated with the asymmetric and symmetric stretching vibrations of the C–O–C ether linkages, respectively. Furthermore, compounds **4l-n** exhibited halogen-associated absorption bands at 1093 cm^{-1} and 1198 cm^{-1} for C–Cl and C–F and at 1082 cm^{-1} and 951 cm^{-1} for C–Br stretching, confirming the presence of halogen substituents in these derivatives and prominent nitro stretches were observed at 1432 cm^{-1} (asymmetric) and 1345 cm^{-1} (symmetric) in the compound **4o**.

The ^{1}H NMR spectra of the synthesized compounds **4a-o** provided structural confirmation through characteristic chemical shifts. A singlet corresponding to the alkene proton was consistently observed in the range of δ 8.02-8.71 ppm, while the amide proton appeared as a distinct singlet between δ 9.70-10.41 ppm in all the compounds. Methoxy protons in compounds **4d-h** appeared as singlets in the range of δ 3.82-3.88 ppm. A methyl proton signal was observed in compound **4i** as a singlet at δ 2.49 ppm and isopropyl group protons appeared at δ 1.23 ppm (doublet, CH_3) and δ 2.92-3.01 ppm (septet, CH) in the compound **4j**. Compound **4k** exhibited a singlet at δ 2.38 ppm corresponding to the N,N-dimethyl protons. Furthermore, hydroxyl protons were identified as singlets in the range of δ 10.17-10.31 ppm in compounds **4b**, **4c**, **4g** and **4h** supporting the presence of phenolic OH functionalities.

The ^{13}C NMR spectra of the synthesized compounds **4a-o** further validated their proposed structures through the observation of characteristic chemical shifts. Signals in the range of δ 171.00-172.04 ppm were attributed to carboxylic acid while resonances between δ 159.84-161.83 ppm and δ 157.67-158.34 ppm correspond to the phenolic carbon atoms and the amide, respectively. Methoxy carbon atoms present in compounds **4d-h** exhibited chemical shifts within δ 55.57-60.78 ppm. Methyl carbon resonances were observed at δ 26.50 ppm for compound **4i**, δ 23.41 and 33.56 ppm for the isopropyl group in **4j** and δ 39.56 and 39.92 ppm for the N,N-dimethyl moiety in compound **4k**. Substituent specific shifts were also evident, which are explained as for compound **4l** (C–Cl) exhibited a signal at δ 136.99 ppm, **4m** (C–F) at δ 163.50 ppm, **4n** (C–Br) at δ 122.20 ppm and **4o** (C–NO_2) at δ 132.81 ppm indicating the presence of halogen and nitro substituents. Furthermore, the characteristic nitrile carbon appeared as a distinct signal in the range of δ 117.00-117.97 ppm across all cyanoacetylated derivatives.

Mass spectra of the compounds were recorded and the observed peaks corresponding to $(M+H)^+$ and $(M–H)^–$ ions were consistent with the theoretical molecular formulae.

***In vitro* antioxidant activity:** The antioxidant potential of the synthesized compounds **4a-o** was evaluated using both DPPH and nitric oxide radical scavenging assays at a concentration of 100 μM. The data are presented in Table-1. The DPPH radical scavenging assay results revealed that, except compound **4g** (3-OCH_3, 4-OH; 64.23%), all tested compounds exhibited strong *in vitro* antioxidant activity with percentage inhibition ranging from 74.50% to 93.23%. Notably, these compounds outperformed the standard antioxidant ascorbic acid (68.78%) and the reference drug 5-ASA (72.80%). Among the series, compound **4e** (3,4-OCH_3; 93.23%) demonstrated the highest scavenging activity, indicating excellent free radical scavenging potential. The NO radical scavenging assay results indicate that the compounds exhibit promising *in vitro* antioxidant activity in the range of 49.49-72.70%, some of them like **4c** (3,4-OH; 64.20%) and also a few **4b** (4-OH; 70.19%), **4e** (3,4-OCH_3; 72.70%) and **4j** (4-$CH(CH)_3)_2$; 70.43%) exceeding the reference standards ascorbic acid (64.24%) and 5-ASA (70.72%). Among all the compounds, **4e** (3,4-OCH_3; 72.70%) showed the highest nitric oxide radical scavenging activity. These findings highlight the dual *in vitro* antioxidant efficacy of compound **4e** (3,4-OCH_3) across both DPPH and nitric oxide radical assays promising it for further pharmacological investigation [33].

***In vitro* anti-inflammatory activity:** The anti-inflammatory potential of the compounds was assessed using protein denaturation and the HRBC membrane stabilization methods at 100 μM concentration. The results are compared to the standard anti-inflammatory 5-ASA as shown in Table-2. In the protein denaturation assay, which reflects the ability to inhibit heat-induced denaturation of proteins, compound **4l** (4-Cl; 79.27%) demonstrated the highest inhibition among the test compounds followed by compounds **4b** (4-OH; 74.32%), **4c** (3,4-OH; 70.41%) and **4m** (4-F; 65.55%) with moderate inhibition [24]. Although the derivatives were slightly less active than 5-ASA (85.01%), most of them exhibited a good percentage of protection (>50%) in the protein denaturation assay [34]. The HRBC membrane stabilization assay, which evaluates the protection of red blood cells against hypotonicity-induced lysis reveals that several compounds exhibited high levels of protection. Notably, **4m** (4-F; 77.10 %), **4k** (4-$N(CH_3)_2$; 76.14%) [35], **4l** (4-Cl; 75.40%), **4o** (4-NO_2; 75.36%), **4h** (3,5-OCH_3, 4-OH; 74.99%) and **4n** (4-Br; 74.67%) [36], showed protection greater than 5-ASA (74.67%) and all the remaining compounds demonstrated moderate activity ranging between 64.60% and 73.61%.

TABLE-1
In vitro ANTIOXIDANT ACTIVITY OF COMPOUNDS (**4a-o**) AGAINST DPPH AND NITRIC OXIDE RADICAL SCAVENGING METHODS

Compd.	% Inhibition of DPPH at 100 μM*	% Scavenging by nitric oxide radical at 100 μM*
4a	77.14	62.45
4b	88.99	70.19
4c	86.24	64.20
4d	92.17	63.18
4e	93.23	72.70
4f	79.15	50.55
4g	64.23	57.88
4h	85.19	49.49
4i	88.04	68.04
4j	87.20	70.43
4k	74.50	61.28
4l	87.30	57.19
4m	92.28	51.28
4n	90.58	56.10
4o	85.82	62.45
5-ASA	72.80	70.72
Ascorbic acid	68.78	64.24

*Average of triplicate measurement

TABLE-2
In vitro ANTI-INFLAMMATORY ACTIVITY OF COMPOUNDS (**4a-o**) BY PROTEIN DENATURATION AND HRBC MEMBRANE STABILIZATION METHODS

Compd.	% Inhibition of protein denaturation at 100 μM*	% Protection in HRBC membrane stabilization method at 100 μM*
4a	59.01	64.60
4b	74.32	68.05
4c	70.41	70.11
4d	52.63	71.86
4e	48.17	71.03
4f	61.40	73.61
4g	52.07	68.51
4h	56.38	74.99
4i	45.45	73.15
4j	49.12	71.54
4k	53.83	76.14
4l	79.27	75.40
4m	65.55	77.10
4n	60.05	74.67
4o	59.57	75.36
5-ASA	85.01	74.67

*Average of triplicate measurement.

These findings suggest that the cyano group introduced *via* cyanoacetylation and Knoevenagel condensation, is the key driver of the enhanced activity [37,38]. Its strong electron-withdrawing effect creates a push-pull system that lowers phenolic O-H bond energy, stabilizes the phenoxy radical through extended Ar-CH=C(CN)-C=O conjugation and improved DPPH and NO scavenging (ex: **4e**). The α-cyanoenone also traps radicals, optimizes the lipophilicity/polarity and improves protein or membrane binding leading to superior protein denaturation inhibition and HRBC stabilization transforming novel derivatives into highly potent dual antioxidant and anti-inflammatory agents compared to 5-ASA as shown in Fig. 1.

Drug-likeliness: The *in silico* molecular properties of the synthesized compounds **4a-o** were analyzed to assess potential drug-likeness and structural diversity and are presented in Table-3. The studies revealed that the dataset is well-structured and largely compliant with drug-likeness criteria, specifically Lipinski's rule of five (MLogP ≤ 5, MW ≤ 500, HBA ≤ 10, HBD ≤ 5, n Violations = 0). The MLogP values ranged from 2.13 to 4.61 falling within the desirable range for oral bioavailability (MLogP < 5). All compounds had topological polar surface area (TPSA) values below 160 $Å^2$ suggesting good ability for gastrointestinal absorption. Molecular weight values ranged from 308 to 398.37 remaining within Lipinski's rule of 500. The number of hydrogen bond donors (HBD) and acceptors (HBA) ranged from 3-5 and 6-9 respectively, aligning with common thresholds for good oral bioavailability. All compounds showed zero violations of drug-likeness rules and varied in number of rotatable bonds (nrotb) from 4 to 7, indicating a range of molecular flexibility. Overall, the data reflect a well-designed library with varied lipophilicity and flexibility, suitable for structure–activity relationship (SAR) studies.

The PASS analysis and Osiris property explorer data of the title compounds **4a-o** and a reference compound (5-ASA) based on their anti-inflammatory activity and potential toxicity profiles are presented in Table-4. Among the compounds, **4j** (4-$CH(CH_3)_2$) exhibits the highest predicted anti-inflam-

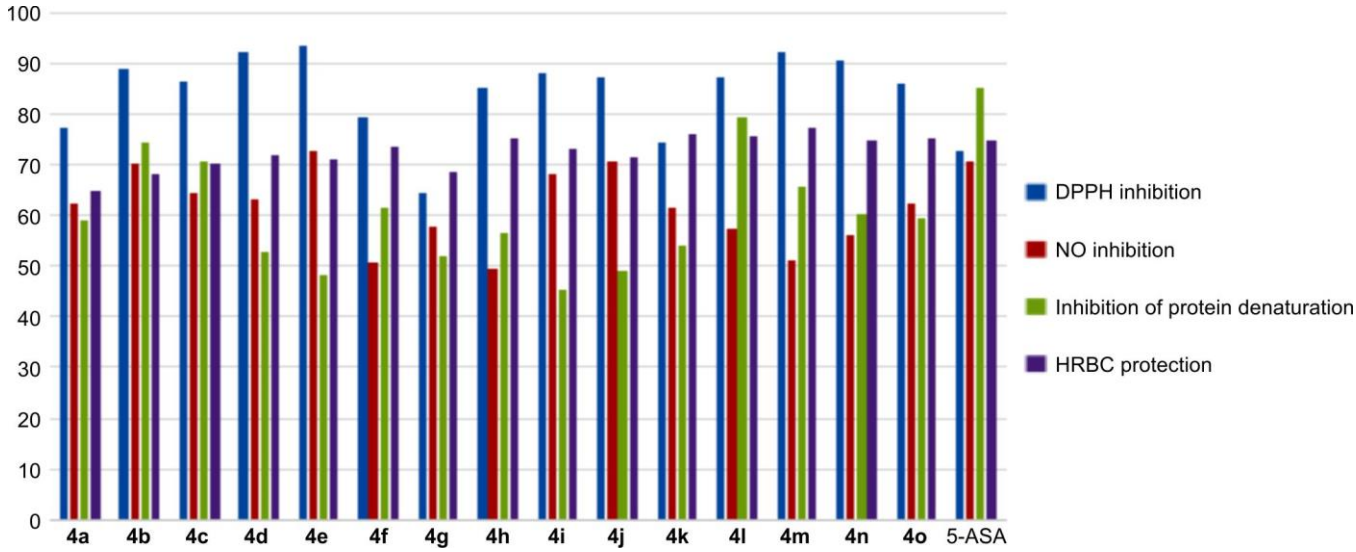


Fig. 1. *In vitro* antioxidant and anti-inflammatory activities of the compounds (**4a-o**)

TABLE-3
PHYSICO-CHEMICAL PROPERTIES OF COMPOUNDS (**4a-o**) GENERATED BY MOLINSPIRATION CHEMINFORMATICS

Compd.	Milog P	TPSA	n atoms	M.W.	HBA	HBD	n Violations	n rotb	Volume
4a	3.10	110.42	23	308.29	6	3	0	4	266.13
4b	2.62	130.65	24	324.29	7	4	0	4	274.15
4c	2.13	150.87	25	340.29	8	5	0	4	282.70
4d	3.16	119.65	25	338.32	7	3	0	5	291.68
4e	2.75	128.88	27	368.35	8	3	0	6	317.23
4f	2.73	138.12	29	398.37	9	3	0	7	342.77
4g	2.44	139.88	26	354.32	8	4	0	5	299.70
4h	2.45	149.11	28	384.34	9	4	0	6	325.25
4i	3.55	110.42	24	322.32	6	3	0	4	282.69
4j	4.61	110.42	26	350.57	6	3	0	5	316.08
4k	3.20	113.66	26	351.36	7	3	0	5	312.04
4l	3.78	110.42	24	342.74	6	3	0	4	279.67
4m	3.26	110.42	24	326.28	6	3	0	4	271.06
4n	3.91	110.42	24	387.19	6	3	0	4	284.02
4o	3.06	156.24	26	353.39	9	3	0	5	289.47

MW = molecular weight; HBA = number of H-bond acceptors; HBD = number of H-bond donors; n rotb = number of rotatable bonds; TPSA = topological polar surface area.

TABLE-4
PASS PREDICTED ANTI-INFLAMMATORY ACTIVITY AND TOXICITY RISK ASSESSMENT BY OSIRIS PROPERTY EXPLORER FOR THE COMPOUNDS (**4a-o**)

Compound	Probable activity value; anti-inflammatory activity	Mutagenic	Tumerigenic	Irritant	Reproductive effective
4a	0.507	Low risk	Low risk	Low risk	Low risk
4b	0.509	Low risk	Low risk	Low risk	Risk
4c	0.526	Low risk	Low risk	Low risk	Low risk
4d	0.492	Low risk	Low risk	Low risk	Low risk
4e	0.496	Low risk	Low risk	Low risk	Low risk
4f	0.493	Low risk	Low risk	Low risk	Low risk
4g	0.509	Low risk	Low risk	Low risk	Low risk
4h	0.503	Low risk	Low risk	Low risk	Low risk
4i	0.511	Low risk	Low risk	Low risk	Low risk
4j	0.578	Low risk	Low risk	Low risk	Low risk
4k	0.435	Risk	Risk	Low risk	Low risk
4l	0.498	Low risk	Low risk	Low risk	Low risk
4m	0.519	Low risk	Low risk	Low risk	Low risk
4n	0.401	Low risk	Low risk	Low risk	Low risk
4o	0.420	Risk	Risk	Low risk	Low risk
5-ASA	0.588	Medium risk	Risk	Low risk	Low risk

matory activity (Pa = 0.578) followed by **4c** (3,4-OH; 0.526) and **4m** (4-F; 0.519), indicating strong potential as anti-inflammatory agents. All compounds, except **4b** (4-OH), **4k** (4-N(CH_3)$_2$) and **4o** (4-NO_2) reported favourable toxicity profiles, suggesting good safety. Compound **4b**(4-OH), while showing a decent activity score (0.509), has a reproductive toxicity alert, making it less desirable. Compounds **4k** (4-N(CH_3)$_2$) and **4o** (4-NO_2) stand out with toxicity for mutagenicity and tumorigenicity, raising concerns about their mutagenic and carcinogenic potential despite moderate activity (0.435 and 0.420, respectively). Simple replacement of N atom with -CH group in compound **4j** (4-CH(CH_3)$_2$) results in the most promising candidate due to their higher Pa values and favourable toxicity profile than that of the standard 5-ASA.

Conclusion

A series of novel 5-aminosalicylic acid (5-ASA) derivatives (**4a-o**) were synthesized *via* cyanoacetylation followed by Knoevenagel condensation and confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry. They exhibited promising *in vitro* antioxidant and anti-inflammatory activities, with structure–activity relationships revealing clear substituent effects. Electron-donating group 3,4-dimethoxy substitution in compound **4e**, significantly enhanced dual radical-scavenging potency (DPPH and NO), greater than both ascorbic acid and 5-ASA due to improved stabilization of the phenoxy radical through resonance and hyperconjugation. Halogen substituents such as the *para*-chloro derivative **4l** displayed the strongest inhibition of protein denaturation, while the *para*-fluoro derivative **4m** provided the most effective HRBC membrane stabilization, highlighting the beneficial role of moderately electronegative and small-sized halogens in membrane-protective effects. PASS analysis further supported **4j** (isopropyl), **4c** (catechol) and **4m** (fluoro) as the safest and most promising candidates. These findings demonstrate that strategic incorporation of electron-donating methoxy groups and appropriately positioned halogens can substantially improve both antioxidant and anti-inflammatory profiles of 5-ASA derivatives suggesting for advanced studies in inflammatory models.

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CONFLICT OF INTEREST

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

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