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Synthesis, characterization and biological evaluation of chromone-salicylhydrazide hybrid molecules

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Abstract: **Background**: Chromone and salicylhydrazide are important scaffolds with multiple biological activities. The molecular hybridization of these scaffolds may lead to the synthesis of new molecules with enhanced pharmacological activities and better drug-like properties. Studies on chromone-salicylhydrazide hybrids combined with antioxidant, anti-inflammatory, cytotoxic potential and drug-like properties evaluations are limited. **Objectives**: The study aimed to synthesize, characterize and evaluate the biological potential of chromone-salicylhydrazide-hybrid molecule as anti-inflammatory, antioxidant, cytotoxic and *in-silico* drug-like properties. **Methods**: The chemical synthesis was performed in two steps. In the first step, salicylhydrazide was synthesized from methyl salicylate. In the second step, salicylhydrazide was reacted with chromones to produce the hybrid molecules 5a-5c. Compounds 5a-5c were characterized by using the Fourier transform infrared spectroscopy (FTIR), Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), Carbon -13 nuclear magnetic resonance spectroscopy (^{13}C-NMR) and mass spectroscopic (MS) techniques. Derivatives 5a-5c were evaluated for *in-vitro* antioxidant, anti-inflammatory and cytotoxic potential. The drug-like characteristics were assessed by *in-silico* analysis. **Results**: Chromone-salicylhydrazide molecules exhibited promising biological activity. Compound 5b (IC_{50} = 0.546 µg/mL) presented excellent antioxidant activity. Compound 5a (59-61% inhibition) exhibited excellent anti-inflammatory potential. The cytotoxic study against the normal cell line (3T3) showed less toxicity (31-47% inhibition). Compounds 5a-5c presented drug like properties by complying the Lipinski and Veber rules. **Conclusion**: Chromone-salicylhydrazide molecules manifested excellent antioxidant and anti-inflammatory potential with low toxicity toward the normal cell line along with suitable drug-like properties. Therefore, they are promising biologically active agents for further investigations.

Keywords: Characterization; Drug-likeness; Hybridization; *In-vitro* evaluation; Synthesis

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INTRODUCTION

Chromone is an oxygen-containing heterocyclic compound and is widely distributed in nature (Yang *et al*., 2023). Chromones are found in various plants and are biosynthesized by various pathways. Apigenin and luteolin (Fig. 1) are known secondary metabolites of plants that consist of chromone scaffolds (Amen *et al*., 2021). Chromones are found in many products of pharmaceutical importance and perform vital activities. Chromone-containing molecules have antimalarial (Watanabe *et al*., 2023), antibacterial (Kanzouai *et al*., 2023), antiviral (TN *et al*., 2023), antioxidant (Maicheen *et al*., 2023), anti-diabetic (Fan *et al*., 2023a), anticancer (Chen *et al*., 2023), anti-inflammatory (Zapata Lopera *et al*., 2022), anti-allergic and antiulcer (Danai *et al*., 2021) properties. Chromone is less toxic to animals and human beings and hence attractive for new drug development (Benny *et al*., 2022). Chromone ring is present in officially approved medicinal products like flavoxate and nedocromil. The chromone scaffold is a condensed ring of benzene and a γ-pyrone heterocycle (Fan *et al*., 2023b). Chromones have the tendency to react with various nucleophiles thus facilitating the synthesis of structurally diverse and biologically active derivatives (Abdel-Megid *et al*., 2026). Chromone has the tendency to react with different types of biological targets in the body (Kaishap *et al*., 2025). Therefore, the chromone scaffold is an excellent starting point for the design and development of new therapeutic agents.

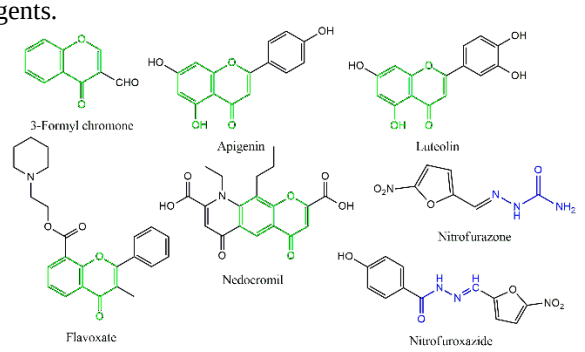

Fig. 1: Chromones and hydrazide based natural products and official approved drugs.

Hydrazides are an important class of functional groups in organic and medicinal chemistry that comprise the R-CO-NH-NH_2 group. Hydrazide possesses two interlinked nucleophilic nitrogen atoms and the carbon-nitrogen bond is conjugated with the carbonyl group (Teixeira et al., 2025). Hydrazide is an important precursor for the synthesis of bioactive compounds. Nitrofurazone and nifuroxazide (Fig. 1) are the hydrazide-containing drug molecules (Başaran *et al*., 2022). Combining the hydrazide

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group with other functional groups produced molecules with interesting biological properties. The conversion of the free amino group of these compounds into hydrazone gives them metabolic safety (Liao *et al*., 2026). Hydrazones have different biological properties like anti-inflammatory, antioxidant, anticancer, antimicrobial and cardioprotective (Borik, 2025; Khattar *et al*., 2023). The imine group (CH=N) can adhere to the active site of enzymes by hydrogen bonding and pi-pi ($\pi - \pi$) interactions (Ribeiro and Correia, 2024). The molecular hybridization is a rational drug design technique that merges two molecules to form a new core. This strategy is used to increase biological activity, selectivity and physicochemical properties along with reduced side effects. Molecular hybridization is a valuable modification tool to design and develop new drugs (de Sena Murteira Pinheiro *et al*., 2024; Morais, 2024). Several chromone-based hydrazide and hydrazone derivatives have been synthesized and evaluated for a variety of biological activities. El-Shaaer *et al*. synthesized chromone-hydrazide (Fig. 2) derivatives using para-toluenesulfonic acid (PTSA) as a catalyst and investigated their antimicrobial activity (El-Shaaer *et al*., 1998). Fan *et al*. reported chromone-benzohydrazides as glucosidase inhibitors (Fan *et al*., 2023c). Zareen *et al*. synthesized chromone-salicylhydrazide derivatives as acetylcholinesterase (AChE) inhibitors (Zareen *et al*., 2026). Galarraga *et al*. developed chromone-hydrazone derivatives as potential antiparasitic agents and Sant and coworkers reported chromone-hydrazone derivatives with anticancer activity (Galarraga *et al*., 2023; Sant *et al*., 2026). Despite these advances, the synthesis of chromone-salicylhydrazide hybrid molecules having substituents on the chromone scaffolds along with combined antioxidant, anti-inflammatory and cytotoxic properties has not been reported previously. Therefore, the present study was planned to synthesize and characterize substituted chromone-salicylhydrazide hybrid molecules. The synthesized derivatives were evaluated as anti-inflammatory, antioxidant and cytotoxic agents and the drug-like properties were assessed by *in-silico* approaches. The findings of this study may lead to the identification of multifunctional lead compounds for new drug discovery.

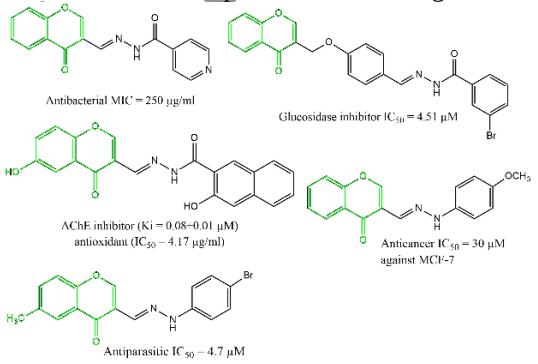

Fig. 2: Chromones-hydrazide/hydrazone as biologically active agents.

MATERIALS AND METHODS

Chemicals and solvents

Chemicals and solvents were all bought from commercial providers for the experiments. 3-Formylchromone, 6-fluoro-3-formylchromone, 6-methyl-3-formylchromone, methyl salicylate, hydrazine hydrate, ethanol, methanol, acetic acid, chloroform, dimethyl sulfoxide (DMSO), Silica gel (0.25 mm, $60F_{254}$) plate for thin layer chromatography (TLC).

Instruments

Analytical weighing balance (JJ224BF), Melting point apparatus digital (Stuart SMP-10), UV Lamp (254-365 nm), Vacuum rotary evaporator, Hot plate magnetic stirrer (MSHP-1/02), Oven, Ultraviolet-visible Spectrophotometer (UV-Vis spectrophotometer) (Agilent Technologies), Fourier transform infrared spectrometer (FTIR Perkin Elmer), NMR, 400 MHz spectrophotometer for ^{1}H-NMR and ^{13}C-NMR analysis, Mass spectrometer (JEOL-600H-1) for MS analysis.

Synthesis of chromone-salicylhydrazide derivatives

Step-1: Synthesis of intermediate salicylhydrazide

In the first step, 0.38 g (2.5 mmole) of methyl salicylate (1) was treated with 0.25 g (5 mmole) of hydrazine monohydrate (2) in 20 mL ethanol as solvent (Scheme 1). The reaction was refluxed at 80-90 ºC for 16 hours (hrs.) with continuous stirring using reflux (Ali *et al*., 2024). The reaction was tracked by TLC using chloroform and methanol (4.7: 0.3 mL) as the mobile phase. After the completion, the contents of the flask were cooled at room temperature. The precipitates that appeared in the reaction mixture were filtered and dried at room temperature. The intermediate product (3) was re-crystallized by dissolving in 20 mL ethanol, then evaporated to half of the volume through a rotary evaporator and was allowed to settle down overnight. The obtained white crystals were filtered and dried again to produce the compound 3. Melting point 147-152 °C, retention factor (Rf) value 0.22. FTIR (cm^{-1}): 3312 (NH stretching amine), 3193 (CH stretching alkene), 1605 (C=C stretching), 1490 (C=C stretching), 1486 (C=O stretching), 1353 (C-N stretching), 1237 (C-O), 948 (OH bending), 793 (NH bending), 744 (CH bending). The FTIR spectrum of the intermediate product was in accordance with the previous reported synthesis and characterization (Arunagiri *et al*., 2025). Therefore, the intermediate product was considered sufficiently pure and used in the second step reaction.

Step-2: General methodology for the synthesis of chromone-salicylhydrazide hybrids (5a-5c)

The salicylhydrazide (0.01 moles) and chromones (0.01 moles) in equimolar quantities were weighed and shifted into a round-bottom flask. Transferred 20 mL absolute ethanol as solvent and two to three drops of ethanoic acid as a catalyst (Fan *et al*., 2023c). The reaction was heated by reflux condensation at 80-90 ºC for 10 hrs with continuous stirring. The reaction progress was checked by

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using the TLC while using chloroform and methanol (4.7:0.3 mL) as the mobile phase. After the reaction, the contents of the flask were cooled down at room temperature. The precipitates appeared in the flask, then precipitates were filtered and dried. The dried product was re-crystallized by dissolving in 20 mL ethanol, then evaporated to half of the volume and was allowed to cool down overnight. The crystals that appeared were filtered and dried again. Then TLC was performed to calculate the Rf value. Then melting point was determined and the percentage yield was calculated.

Synthesis of (E)-2-hydroxy-N'-((4-oxo-4H-chromen-3-yl)methylene)benzohydrazide (5a)

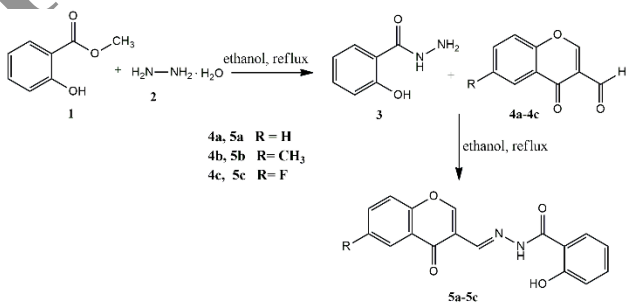
The 0.07 g (0.46 mmole) salicylhydrazide (3) reacted with 0.08 g (0.46 mmole) 3-formyl chromone (4a) in 20 mL ethanol to yield 0.07 g (49.6%) of compound 5a. Melting point 255-260 °C, Rf value 0.25. FTIR (cm^{-1}): 3244 (NH stretching, secondary amine), 1654 (CH=N double bond), 1619 (C=C double bond), 1558 (C=O double bond), 1455 (C=C stretching), 1362 (CH bending alkene), 1213 (ether linkage), 1123 (C-N stretching), 1097 (ether linkage), 944 (OH bending), 754 (CH bending mono-substituted aromatic). ^{1}H-NMR (400 MHz, DMSO): δ 6.93-7.00 (m, 2H, aromatic H), 7.46 (t, 1H, *J* =8.0, aromatic H), 7.57 (t, 1H, *J* =12.0, aromatic H), 7.75 (d, 1H, *J* =8.0, aromatic H), 7.87 (t, 1H, *J* =8.0, aromatic H), 7.93 (dd, 1H, *J*1 =8.0, *J*2 =4.0, aromatic H), 8.15 (d, 1H, *J* =8.0, aromatic H), 8.64 (CH-O), 8.86 (CH=N), 10.89 (1H, CONH), 11.8 (1H, phenolic OH). ^{13}C-NMR (100 MHz, DMSO): δ ppm, 115.39, 117.74, 119.24, 119.7, 125.69, 126.64, 128.82, 129.25, 134.40, 135.24, 141.84 (aromatic carbons), 155.32 (CH=N), 156.26 (CH-O), 158.98 (CH-O), 159.76 (Aromatic carbon, C-OH), 166.27 (C=O, CONH), 175.5 (C=O, chromone). Mass/charge (*m/z*) ratio: calculated $C_{17}H_{12}N_2O_4$: 308.29; found 308.20.

Synthesis of (E)-2-hydroxy-N'-((6-methyl-4-oxo-4H-chromen-3-yl)methylene)benzohydrazide (5b)

The 0.08 g (0.52 mmole) salicylhydrazide reacted with 0.10 g (0.52 mmole) 6-methyl-3-formyl chromone (4b) in 20 mL ethanol to produce 0.1 g (59.2%) of compound 5b. Melting point 240-245 °C, Rf value 0.48. IR (cm^{-1}): 3231 (NH stretching, secondary amine), 3080 (CH stretching, alkene), 1641 (CH=N double bond), 1600 (C=C double bond), 1545 (C=O double bond), 1474 (C=C stretching), 1308 (CH bending alkene), 1235 (ether linkage), 1153 (C-N stretching), 1073 (ether linkage), 909 (OH bending), 754 (CH bending mono-substituted aromatic). ^{1}H-NMR (400 MHz, DMSO): 2.45 (s, 3H, CH_3), 6.95 (t, 1H, *J* = 8, aromatic H), 6.97 (d, 1H, *J* = 8, aromatic H), 7.44 (t, 1H, *J* = 7.2, aromatic H), 7.64 (t, 1H, *J* = 8, aromatic H), 7.69 (d, 1H, *J* = 8, aromatic H), 7.88 (dd, 1H, *J* = 8, aromatic H), 7.93 (s, 1H, aromatic H), 8.63 (CH-O), 8.83 (CH=N), 11.85 (1H, NH), 11.95 (1H, phenolic OH). ^{13}C-NMR (100 MHz, DMSO): δ ppm, 20.94 (1C, CH3), 116.08, 117.77, 118.47, 119.03, 119.38, 123.50, 124.92, 128.81, 134.39, 136.26, 136.33 (Aromatic carbons), 142.02 (CH=N), 154.56 (CH-O), 155.18 (CH-O), 159.78 (aromatic carbon, C-OH), 165.44 (C=O, CONH), 175.44 (C=O, chromone). *m/z* ratio: calculated $C_{18}H_{14}N_2O_4$: 322.31; found 322.20.

Synthesis of (E)-N'-((6-fluoro-4-oxo-4H-chromen-3-yl)methylene)-2-hydroxybenzohydrazide (5c)

The 0.08 g (0.53 mmole) of salicylhydrazide reacted with 0.10 g (0.52 mmole) 6-fluoro-3-formyl chromone in 20 mL ethanol to yield 0.09 g (52.6%) of compound (E)-N'-((6-fluoro-4-oxo-4H-chromen-3-yl)methylene)-2-hydroxybenzohydrazide (5c). Melting point 225-230 °C, Rf value 0.31. FTIR (cm^{-1}): 3080 (NH stretching, secondary amine), 1647 (CH=N double bond), 1600 (C=C double bond), 1543 (C=O double bond), 1455 (C=C stretching), 1306 (CF stretching alkene), 1241 (ether linkage), 1153 (C-N stretching), 1038 (ether linkage), 959 (OH bending), 734 (CH bending). ^{1}H-NMR (400 MHz, DMSO): δ 6.95 (t, 1H, *J* = 8, aromatic H), 6.97 (d, 1H, *J* = 8, aromatic H), 7.44 (t, 1H, *J* = 8.0, aromatic H), 7.75-7.89 (m, 4H, aromatic H), 8.62 (1H, CH-O cyclic), 8.89 (1H, CH=N), 11.82 (1H, NH), 11.97 (1H, phenolic OH). ^{13}C-NMR (100 MHz, DMSO): δ ppm, 110.22, 116.12, 117.77, 118.10, 119.38, 122.10, 123.19, 123.45, 125.09, 128.83, 134.41 (aromatic carbons), 141.55 (CH=N), 152.7 (CH-O), 155.59 (CH-O), 159.74 (aromatic carbon, C-OH), 160.98 (C-F), 165.47 (C=O, CONH), 174.91 (C=O, chromone). *m/z* ratio: calculated $C_{17}H_{11}FN_2O_4$: 326.28; found 326.30.



Scheme 2: Reaction pathway for the synthesis of chromone-salicylhydrazide derivatives.

Evaluation of antioxidant potential

The *in-vitro* antioxidant capacity of the chromone-salicylhydrazide derivatives (5a-5c) was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging protocol (Zandvakili *et al*., 2022). In this method, 1 mL of 0.004% DPPH in methanol (freshly prepared) was mixed to 3 mL of the tested sample in different concentration (1000-0.9 µg/mL) and the mixture was kept for 30 minutes (mins.) in the dark. Then absorbance was taken at 517 nm on UV-Vis spectrophotometer. The solution without a tested sample was used as a control and the standard of study was ascorbic acid (vitamin C), used with the same concentrations as samples. All the tests were carried out in triplicate. The following formula calculated the percentage DPPH inhibition:

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%age DPPH Inhibition = absorbance of control – absorbance of sample/ absorbance of control × 100

Evaluation of anti-inflammatory activity

The *in-vitro* anti-inflammatory activity of derivatives 5a-5c was evaluated by following two methods.

Bovine serum albumin (BSA) assay

Firstly, 1% BSA solution was prepared by adding 1 g BSA powder to 100 mL of distilled water. Then solutions of chromone derivatives (1000-31.25 µg/mL) were prepared in glass vials by using DMF as solvent. The control solution was prepared by adding dimethyl formamide (DMF) (0.05 mL) to 0.45 mL of BSA. The standard solution was prepared by adding 0.05 mL of naproxen sodium solution to 0.45 mL of BSA. In-order to make the test compound formulations for UV absorbance, 0.05 mL of sample solution was added to 0.45 mL of BSA using a micro pipette. All the test tubes were incubated at 37 °C for 20 mins. followed by heating in a water bath at 70°C for 15 mins. and then cooled to room temperature. After this, 2.5 mL of phosphate buffer was added to each solution. The final volume of all solutions was 3mL. Finally, the absorbance of all samples was noted at 660 nm wavelength using a UV-Vis spectrophotometer. The percentage (%age) inhibition by chromone derivatives was calculated by the following formula (Ouadghiri *et al*., 2026).

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Egg albumin assay

Firstly, 1% egg albumin solution was prepared by adding 1 mL of fresh hen's egg albumin to 100 mL of distilled water. Then solutions of chromone derivatives 5a-5c (1000-31.25 µg/mL) were prepared in DMF. The control solution was prepared by adding DMF (2 mL) to 0.2 mL of egg albumin and 2.8 mL of phosphate buffer. The standard solution was prepared by adding 2 mL of naproxen sodium (1 mg/mL) solution in 0.2 mL of egg albumin and 2.8 mL of phosphate buffer. To make the sample solution, added 2 mL of the sample (chromone derivatives) to 0.2 mL of egg albumin solution and 2.8 mL of buffer using a micropipette. The final volume of all solutions was 5 mL. All the test tubes were incubated at 37 °C for 20 mins. then heated in a water bath at 70 °C for 15 mins. and then allowed to cool to room temperature. Finally, absorbance of the above-mentioned formulations was noted at 660 nm wavelength using a UV-Vis spectrophotometer. The percentage inhibition by chromone derivatives was calculated by above formula (Das *et al*., 2022).

Evaluation of cytotoxicity of compounds

The *in-vitro* cytotoxic assay of 5a-5c derivatives was performed by using the standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay in 96-well microplates to determine the cytotoxic effect on cultured mammalian cells (Khalef *et al*., 2024). The mouse fibroblast cell line (3T3) was grown in Dulbecco's Modified Eagle Medium (DMEM), containing 5% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin in 75 cm^2 flasks. The cells were incubated at 37 ^oC in a humidified incubator containing 5% CO_2. The rapidly growing cells were harvested by using 0.25% trypsin and EDTA, collected, counted with hemocytometer and the dilution was done with medium. Cell culture with a concentration of 5×10^3-$5x10^4$ cells/mL was added (100 µL/well) into 96-well plates. The plates were incubated overnight for cell attachment. After overnight incubation, the medium was removed and 200 µL of fresh medium with various concentrations of 5a-5c was added. The untreated cells served as the negative control and vehicle treated cells served as the solvent control. After 48 hrs. of incubation, 200 µL of MTT (0.5 mg/mL) reagent was added and incubation for 4 hrs. During this period, metabolically viable cells reduced the yellow salts into purple formazan crystals. After one-hour of incubation, the MTT reagent was carefully removed without disturbing the crystals. Immediately after, DMSO (100 µL) was pipetted to each well and the plates were shaken for complete dissolution. Measured the absorbance of each well at 540 nm by using a microplate reader. The percent inhibition was calculated as follows:

Percentage inhibition = 100- (mean O.D (optical density) of test compounds – mean O.D of control)/ (mean of O.D of standard – mean O.D of control) × 100. Where O.D = optical density

Statistical analysis

The data was presented as mean and standard deviation of triplicate values. Graphpad Prism software was used for statistical analysis. The data was analyzed through the two-way ANOVA to evaluate the effect of concentrations and compounds on percentage inhibitions. A *p* -value less than 0.05 was considered significant.

Evaluation of drug-like properties

The drug-like features of chromone-salicylhydrazide hybrids (5a-5c) were evaluated by online Swiss ADME software. The physicochemical and pharmacokinetic characteristics of compounds were analyzed. The Lipinski rule of five (RO5) and the Veber rule were also applied to predict the drug-like properties.

RESULTS

Chemistry

The chemical synthesis of derivatives was achieved in two steps. The synthesis of the intermediate product was confirmed by melting point and TLC. Then it was analyzed through FTIR to characterize the functional groups. The salicylhydrazide showed NH stretching (3300-3200 cm^{-1}), CH stretching (2900 cm^{-1}), C=O stretching (1400-1500 cm^{-}

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[1]), NH bending (793 cm^{-1}), OH bending (948 cm^{-1}) peaks in the FTIR spectrum. Final compounds (5a-5c) were produced in moderate yield (50-60%). Compounds 5a-5c were pure on the TLC and showed single spots. The characterization of final compounds was performed by FTIR, ^{1}H and ^{13}C-NMR as well as MS analysis. The FTIR spectra of final compounds showed CH=N stretching in the region of 1654-1641 cm^{-1}, which indicated the coupling of two reactants. Similarly, other important functional groups like carbonyl group, ether linkage, substituted aromatic ring were characterized through FTIR analysis. The ^{1}H-NMR spectra of compounds 5a-5c showed downfield shifts (10-12 ppm) for NH and phenolic protons. The aromatic protons appeared in the form of multiplets between 7 ppm and 8 ppm. The ^{13}C-NMR spectra showed aromatic carbons between 116 and 136 ppm. Two carbonyl carbons were shifted downfield and appeared between 165 and 176 ppm. The mass spectra of 5a, 5b and 5c presented molecular ion peaks at m/z ratios 308.20, 322.2, 336.30 respectively and were almost equal to the calculated values. The purity and structural integrity of compounds were confirmed by melting points, NMR and MS analysis.

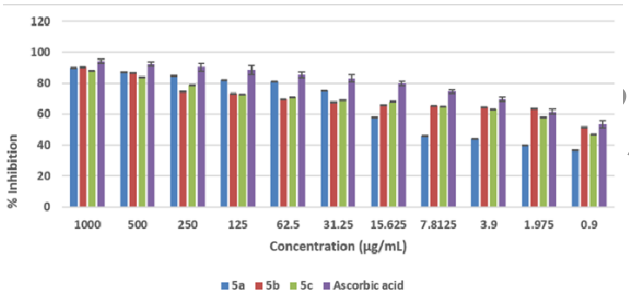

Fig. 3: Antioxidant activity of derivatives 5a-5c (data is presented as mean ± standard deviation of three replicates. Statistical analysis performed by two-way ANOVA. The % inhibition differed significantly among groups and increased significantly with concentrations, $p < 0.0001$)

Evaluation of antioxidant activity

The antioxidant activity of chromone-salicylate hybrids (5a-5c) was evaluated by *in-vitro* DPPH assay at the concentration of 1000 to 0.9 µg/mL (Fig. 3). Derivatives 5a-5c exhibited excellent antioxidant activity (IC_{50} = 0.546-5.241 µg/mL) in comparison to the standard drug ascorbic acid (IC_{50} = 0.20 µg/mL). The half maximum inhibitory concentration (IC_{50}) value represented the concentration needed to produce 50% of maximum biological activity (or 50% inhibition). It was the quantitative measure of the potency of a substance and was primarily defined through *in-vitro* models (Van Truong *et al*., 2025). This value was an important parameter to assess the effectiveness of potential therapeutic compounds. It enabled researchers to compare different compounds and make a decision about their efficacy. A lower IC_{50} value indicated greater biological activity because a small amount of the compound was required to produce the biological activity. Therefore, compounds with a lower IC_{50} value exhibited stronger biological activity (Sánchez-Díez *et al*., 2025). Compound 5b (IC_{50} = 0.546 µg/mL) appeared as the most potent antioxidant derivative. Compound 5b holds a methyl group at position 6 of the chromone core. Compound 5c, having a fluorine atom, presented decreased antioxidant activity (IC_{50} = 0.83 µg/mL).

Evaluation of anti-inflammatory activity

The *in-vitro* anti-inflammatory activity of chromone-salicylhydrazide was performed by BSA and egg albumin assay (1000-31.25 µg/mL). Compound 5a (61-59% inhibition) was found to be the most active anti-inflammatory agent at the concentration of 1 mg/mL (Figs. 4 & 5) in comparison to the standard drug naproxen sodium (92-94% inhibition). Compound 5a has un-substituted chromone ring attached to the salicylhydrazide. Derivatives 5b and 5c were found to be less active in this series.

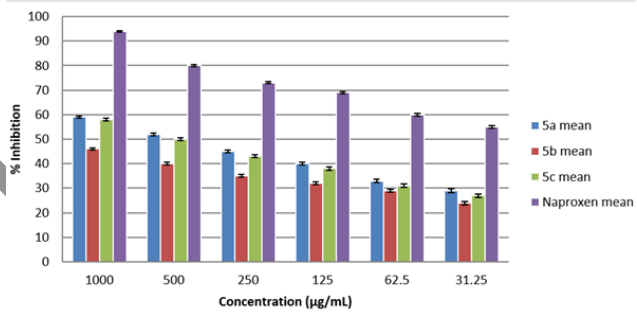

Fig. 4: Anti-inflammatory activity of 5a-5c by BSA **(**data is expressed as mean ± standard deviation of three replicates. Statistical analysis was performed by two-way ANOVA. The % inhibition or dose-response pattern differed significantly among groups and increases significantly with concentrations $p < 0.0001$).

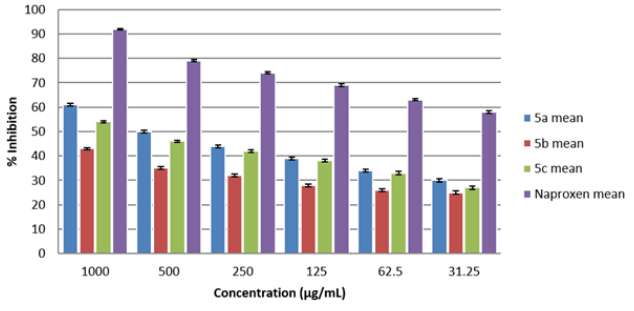

Fig. 5: Anti-inflammatory activity of 5a-5c by egg albumin assay (The % inhibition or dose-response pattern differed significantly among groups and increases significantly with concentrations $p < 0.0001$).

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Table 1: Drug likeness properties of chromone-salicylhydrazide derivatives where BBB=blood brain barrier; Pgp= p-glycoprotein; PAINS: pan assay interference compounds; LLV= lean likeness violation; BS= bioavailability score.

Comp.	5a	5b	5c	Normal value
CSp3	0	0.06	0	0.25-0.1
HBD	2	2	2	<5
HBA	5	5	6	<10
logP	1.72	2.21	1.88	<5
TPSA (Å)	91.9	91.9	91.9	<140
nROT	4	4	4	<10
logS	-3.8	-4.1	-3.96	-4 - -6
MW	308.29	322.31	326.28	<500 Dalton
MR	85.68	90.64	85.63	40-130
BBB	No	No	No	
Pgp. substrate	No	No	No	< 0.5
PAINS alert	No	No	No	0 alert
LLV	0	0	0	
BS	0.55	0.55	0.55	Moderate to good oral activity
SA	3.07	3.18	3.03	3-4 normal/ average

Evaluation of cytotoxic activity

The *in-vitro* cytotoxicity of 5a-5c was evaluated by MTT 96-well microtiter plate assay on the mouse fibroblast (3T3) cell line. The results showed that derivatives (5a-5c) are less cytotoxic (Fig. 6) towards the tested cell line in comparison to doxorubicin (95.14% inhibition; IC_{50} = 0.24±0.13 µM) as a standard drug. Compound 5a (47.12% inhibition) was the most cytotoxic agent and it has an unsubstituted chromone ring. Derivative 5b was the least cytotoxic compound in this series.

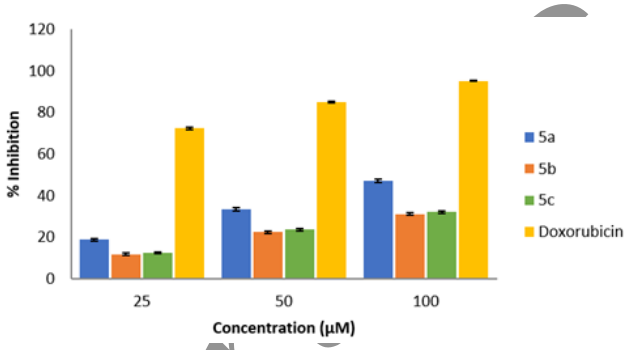

Fig. 6: Cytotoxic activity of 5a-5c by MTT assay (data is displayed as mean ± standard deviation (n = 3) and statistical analysis was carried by two-way ANOVA. The % inhibition differed significantly among groups and increased significantly with concentrations $p < 0.0001$).

Assessment of drug likeness

The evaluation of drug likeness of chromone-salicylhydrazide hybrids showed that derivatives 5a-5c met the criteria of Lipinski rule of five (RO5) that includes molecular weight (MW), partition coefficient (logP), hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). The Veber rule was also obeyed with no violation like the number of rotatable bonds (nROT), topological polar surface area (TPSA) (Caminero Gomes Soares *et al*., 2023; Crucitti *et al*., 2024). Furthermore, the molar refractivity (MR), water solubility (logS) and fraction of *Sp3* hybridized carbon atoms (C*Sp3*) and synthetic accessibility (SA) were found to be within acceptable limits (Table 1).

DISCUSSION

The chromone-salicylhydrazide hybrid molecules were synthesized by a two-step reaction scheme. The intermediate salicylhydrazide and final compounds were characterized by spectroscopic techniques (Shin *et al*., 2021). Reactive oxygen species (ROS) are generated in the body as by-products of normal cellular metabolism. The excessive production of ROS has a role in the development of certain diseases such as cancer and neurological disorders. The antioxidant molecules play a defensive role in the body by scavenging the ROS and minimizing the damage caused by the reactive oxygen species (Halliwell, 2024; Shi *et al*., 2022). The analysis of the antioxidant activity of chromone-salicylhydrazide showed that derivative 5b (IC_{50} = 0.546 µg/mL) presented superior antioxidant activity. Compound 5b comprises a methyl-substituted chromone ring attached to the salicylhydrazide. Therefore, the antioxidant activity may be due to the synergistic effect of the phenolic hydroxy group of salicylhydrazide and the electron-donating methyl substituent on the chromone scaffold. The structure-activity relationship (SAR) further showed that replacement of the methyl group by a fluorine atom and the simple hydrogen atom produced less active chromone derivatives. Therefore, the introduction of electron-donating and lipophilic groups at the chromone scaffold produced highly active antioxidant agents and it was consistent with some previous reports (Shatokhin *et al*., 2022). Inflammation is a defensive response produced by

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the body when it is exposed to harmful stimuli like pathogens and tissue injury. The acute inflammation is essential for tissue repair and host defense. The excessive or persistent inflammatory response is harmful to the body and can contribute to the development of various diseases. Therefore, it is of great clinical importance to develop effective anti-inflammatory agents (Shi, *et al*., 2022; Liu, *et al*., 2022; Wang *et al*., 2024). Upon evaluation of *in-vitro* anti-inflammatory activity, derivative 5a (61% and 59% inhibition) emerged as the most active anti-inflammatory agent. Concerning the substituted chromone derivatives, 5c (58% and 54% inhibition) showed greater activity than 5b (46% and 43% inhibition). The SAR studies showed that substitution of a fluorine atom by the methyl group produced less active anti-inflammatory agents (Xing *et al*., 2023). This was in contrast to antioxidant activity, where the electron-donating group showed better activity. The fluorine and halogen substitution can influence the potency, membrane permeability and metabolism of bioactive molecules (Schubert *et al*., 2023). Cytotoxic agents exert toxic effects on living cells by disrupting cellular processes like cell membrane integrity, protein synthesis and metabolic activity, ultimately leading to cell death. The cytotoxicity can be assessed by various *in-vitro* methods and the MTT assay is one of the most widely employed due to its simplicity, reliability and sensitivity. The cell viability and cytotoxicity assays play a crucial role in drug screening and are important to assess the toxic effects of various chemicals. These assays are reliable, sensitive and cost-effective approaches for appraising the safety and biological activity of new synthesized molecules (Khalef *et al*., 2024). The cytotoxicity assay showed that derivatives 5a-5c are less cytotoxic (31.18-47.12% inhibition) towards the normal cell line in comparison to doxorubicin. Among the synthesized derivatives, the methyl carrying chromone derivative 5b (31.18% inhibition) was found least cytotoxic. Therefore, the introduction of small lipophilic and electron donating substituent on the chromone ring leads to the production of less cytotoxic agents (Subramani *et al*., 2024). Further evaluation is required against cancer cell lines to the determine selectivity and therapeutic potential of these derivatives. The assessment of drug-like properties is important to identify the compounds with favorable pharmacokinetic and to eliminate those with undesirable properties at an early stage of drug development. The advancement of computational methodologies and improvement in machine learning have improved the drug likeness screening process significantly (Pantaleão *et al*., 2022). The drug likeness evaluation of derivatives 5a-5c gave a good indication of the drug-like features of these compounds. These compounds presented good oral absorption but were not expected to cross the BBB. They are favorable for peripheral therapeutic applications. The bioavailability score was 0.55 for all three derivatives that supports their potential for oral activity. The predicted logS values ranged from -3.29 to -3.67, hence they are moderately soluble. None of the compounds was a substrate for P-glycoprotein which eliminated the efflux-mediated drug resistance. In addition, these compounds mediated no PAINS alert and hence a low probability of assay interference. These compounds presented no violation of lead likeness and synthetic accessibility was three (Bilidegalu *et al*., 2025). Therefore, they can be modified further and are relatively easy to synthesize.

CONCLUSION

The chromone-salicylhydrazide hybrids were synthesized and characterized by using different spectroscopic techniques. These synthesized derivatives were evaluated for biological potential and drug-like properties. Among the tested compounds, 5b (IC_{50} = 0.546 µg/mL) displayed prominent antioxidant potential. Derivative 5b (31.18% inhibition) was also found least toxic for the normal cell line (3T3) and indicated a better safety profile. Compound 5a (61-59% inhibition) was found to be the most active anti-inflammatory agent. Compounds 5a-5c exhibited excellent drug-like properties like good oral absorption, acceptable physicochemical properties and are excellent lead compounds. Therefore, chromone-salicylhydrazides are promising drug candidates and deserve further investigation as pharmacologically active agents.

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Authors' contributions
Sidra Rafique: Investigation, data curation; Noor ul Amin Mohsin: conceptualization, formal analysis, writing original draft

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Data availability statement
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent to publication
Not applicable. The article does not contain any studies with human participants or animals performed by any of the authors.

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Ethical approval

This study did not involve human or animal subjects, and thus no ethical approval was required.

Conflict of interest

The authors declare no conflict of interest

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