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Scientific paper

Evaluating the Cytotoxic Effects of Some Chlorinated Substituted 2-Amino-3-carbonitrile Chromene Derivatives on 3T3 and MCF-7 Cell Lines

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Abstract

Cancer remains a leading cause of death worldwide, necessitating novel therapeutic approaches. In this study we synthesized and characterized 2-amino-3-carbonitrile chromene derivatives and evaluated their cytotoxic effects on 3T3 and MCF-7 cell lines. Characterization included melting point, IR, NMR, and *elemental analysis***.** Cytotoxicity was assessed via MTT assay, with IC_{50} values calculated, while apoptosis induction was confirmed by flow cytometry using annexin V/propidium iodide staining. Compounds 4f and 4h demonstrated significant cytotoxicity against breast cancer cells, with IC_{50} values of 4.74 and 21.97 μg/mL and selectivity indices of 3.83 and 2.80, respectively. Increased apoptotic cell populations support their pro-apoptotic potential. These findings indicate that the chromene derivatives, synthesized via a one-pot method, may serve as promising candidates for further anticancer drug development.

Keyword: Chromene derivatives, Anticancer, Breast cancer**,** 3T3, MCF-7.

1. Introduction

The global economic burden of cancer is rapidly increasing as populations continue to grow, age, and adopt certain lifestyle behaviors.[1] Current estimates indicate that approximately 20% of the global population may develop cancer over the course of their lives. Moreover, cancer-related mortality affects about one in nine men and one in twelve women. Projections suggest that by the year 2050, the number of newly diagnosed cancer cases worldwide may surpass 35 million annually.[2] Breast cancer remains the most prevalent cancer in women, both in terms of incidence and mortality rates.[3] These incidence rates vary widely across different regions, with certain Asian and African countries reporting rates below 40 per 100,000 women, while in countries like Australia/New Zealand, North America, and parts of Europe, rates exceed 80 per 100,000.[4]

The primary treatment approaches for cancer include surgery, radiation therapy, chemotherapy, and radiotherapy.[5–7] Modern therapeutic strategies additionally encompass hormonal interventions, anti-angiogenesis approaches, stem cell-based therapies, and immunotherapeutic techniques utilizing dendritic cells.[8] In recent years, nanotechnology-based therapies, gene editing approaches,

and tumor microenvironment-modifying agents have emerged as promising modalities to enhance treatment precision and reduce toxicity.[9–11]

Chemotherapy is commonly used to treat various types of cancer, but its toxic side effects lead to a range of complications, such as gastrointestinal problems, immune system suppression, nausea, hair loss, anemia, and damage to organs like the liver and kidneys.[12] A major challenge in cancer therapy lies in the harmful effects of chemotherapeutic agents on normal tissues, which can greatly compromise the effectiveness of treatment.[13,14] Moreover, the development of resistance to chemotherapy agents is a major barrier in cancer therapy. This resistance leads to the treatment becoming ineffective, forcing doctors to increase the drug dosage, which consequently raises toxicity levels and worsens side effects.[15,16]

Several mechanisms have been identified as contributing to drug resistance, with one of the primary causes being the overexpression of ATP-dependent membrane proteins, which are part of the large family of ABC membrane transporters. These transporters play a key role in drug resistance by efficiently transferring drugs out of cancer cells, making them one of the most significant obstacles in cancer treatment.[17] Additionally, tumor heterogeneity, metabolic reprogramming, and ferroptosis resistance also contribute significantly to therapeutic failure.[18]

Thus, the use of novel medications is prompted by the rise of tumor cells that are resistant to traditional chemotherapeutic drugs. A class of organic compounds known as chromenes from the flavonoid family exhibits biological activities, including anti-tumor, anti-leishmanial, and antibacterial effects.[19,20] Recent research on plant-derived flavonoids, including those extracted from hawthorn, highlights their potential in modulating oxidative stress and cancer pathways.[20] These findings have led to the consideration of these substances for evaluation as potential novel medicinal agents.[21,22]

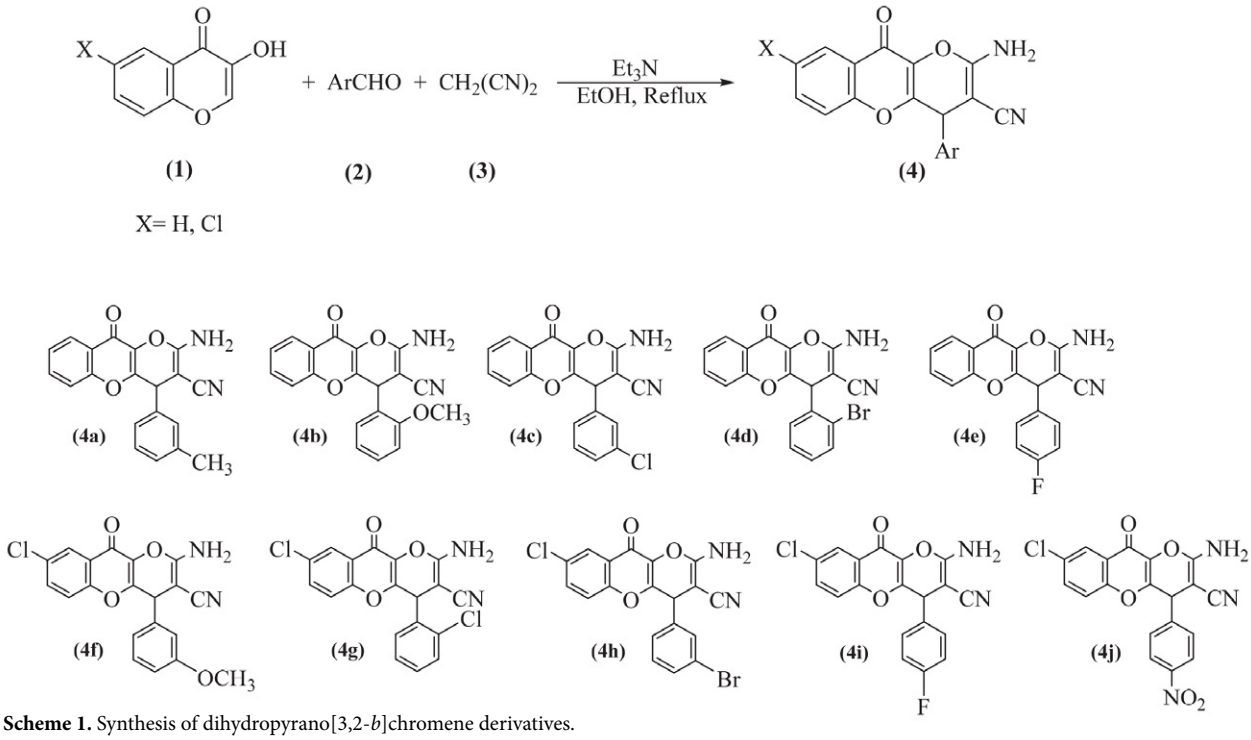
These substances prevent the polymerization of microtubules, resulting in cell cycle arrest and ultimately apoptosis by suppressing cell proliferation and interacting with the colchicine site in tubulin B.[23] These compounds can benefit cancer patients with resistance to conventional antitumor drugs, as they are effective against certain drug-resistant cell types.[24] Some chromene derivatives may also induce ferroptosis or modulate immune responses in the tumor microenvironment.[25–28]

Considering that the anticancer properties of these compounds have been demonstrated through the induction of apoptosis, attempts have been made to synthesize various chromene derivatives.[29,30] The objective of this research was to examine the cytotoxic impact of chlorinated 2-amino-3-carbonitrile chromene derivatives on 3T3 and MCF-7 cell lines. These dihydropyrano chromene derivatives had been synthesized in previous investigations[31–33] and are modified in this study to reach to the novel structures (Scheme 1).

2. Experimental

2. 1. Chemical Synthesis and Structural Characterization

All materials and reagents utilized in this investigation were obtained from commercial suppliers and em-



Scheme 1. Synthesis of dihydropyrano[3,2-*b*]chromene derivatives.

ployed without any further purification. Melting point measurements were carried out using an Electrothermal 9100 apparatus, without applying any correction. Infrared (IR) spectra were recorded on a Bruker Alpha FTIR spectrometer using potassium bromide (KBr) pellets. ^{13}C NMR (75 MHz) and ^{1}H NMR (300 MHz) spectra were obtained on a Bruker AVANCE III 300 MHz spectrometer in dimethyl sulfoxide (DMSO)-d_6, with tetramethylsilane (TMS) as the internal reference. Coupling constants (J) are denoted in hertz (Hz), and chemical shifts (δ) are expressed in parts per million (ppm). Reaction progress was tracked via thin-layer chromatography (TLC) using silica gel GF254 plates on aluminum backing, and spots were visualized under 254 nm UV light. Elemental composition was analyzed using a Heraeus CHN-O-Rapid elemental analyzer.

2. 2. Materials and Methods

Sodium citrate and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA), while DMEM and RPMI were sourced from Biosera (France). Fetal bovine serum (FBS) was acquired from Gibco (USA), and MTT was provided by Melford (UK).

2. 3. General Procedure for the Preparation of Dihydropyrano[3,2-*b*]chromene Derivatives (4a-j)

A 50 mL round-bottom flask fitted with a magnetic stir bar and a reflux condenser was charged with 3-hydroxychromone or 6-chloro-3-hydroxychromone **1** (2 mmol), an aromatic aldehyde **2** (2 mmol), malononitrile **3** (2.1 mmol), and a few drops of triethylamine, all dissolved in 10 mL of ethanol. The reaction mixture was stirred and heated under reflux for one hour. Progress of the reaction was assessed by thin-layer chromatography (TLC), employing a hexane/ethyl acetate mixture as the mobile phase. After completion, the reaction mixture was cooled to room temperature, and the resulting solid was collected by filtration, washed with ethanol, and purified by recrystallization from ethanol to afford the desired product in solid form for further characterization.

2. 4. Cell Lines and Cell Culture

MCF-7 human breast cancer cells and 3T3 mouse embryonic fibroblasts were sourced from the Pasteur Institute of Iran (Tehran, Iran). These cells were cultured in either Roswell Park Memorial Institute (RPMI) 1640 medium or Dulbecco's Modified Eagle's Medium (DMEM), both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2.

2. 5. Assessment of Cytotoxicity Using MTT assay

The cells were plated into 96-well culture plates at a density of 10,000 cells per well for both cell lines and incubated in a CO_2-controlled environment. Treatment was applied the following day in accordance with the experimental protocol. At the specified time intervals, 10 µL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated in the dark for 4 hours. Subsequently, the medium was removed, and the resulting formazan crystals were solubilized in 100 µL of DMSO. Absorbance was recorded at 570 nm using a multi-well plate reader.[34–37] MTT assays were performed in triplicate across three independent biological experiments, and each condition was tested in three technical replicates. Data are presented as mean ± SD. The selectivity index (SI) for chromene compounds against cancer cells was calculated as follows:

2. 6. Apoptosis Analysis Using Flow Cytometry

An annexin V-FITC/PI double staining assay was performed to evaluate apoptosis in MCF-7 cells. The cells were exposed to **4f** and **4h** at concentrations of 4 µg/mL and 21 µg/mL, respectively, for a duration of 24 hours. The FITC-Annexin V apoptosis detection kit (XBIO, Czech Republic) was used according to the manufacturer's instructions. The samples were then analyzed using a BD FACSCalibur flow cytometer to measure the fluorescence intensity.

2. 7. Statistical Analyses

A one-way analysis of variance (ANOVA) was used to compare the results among different experimental groups, followed by Tukey's post hoc test for multiple comparisons. Data are presented as the mean ± standard deviation (mean ± SD), and statistical significance was accepted at $P < 0.05$. All experiments were performed in triplicate.

3. Results and Discussion

In this work, a novel one-pot, three-component synthetic strategy was developed for producing dihydropyrano[3,2-*b*]chromene derivatives (**4a–j**) by reacting 3-hydroxychromones or 6-chloro-3-hydroxychromones (**1**), various aromatic aldehydes (**2**), and malononitrile (**3**) in ethanol using triethylamine (Et_3N) as a catalyst under reflux conditions. The crude products were purified by recrystallization, yielding a series of 2-amino-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile derivatives (**4a–j**) with yields ranging from 81% to 92% (Scheme 1). The structures of the synthesized compounds

were confirmed by elemental analysis, ^{1}H and ^{13}C NMR, and IR spectroscopy.

2-Amino-10-oxo-4-(*m*-tolyl)-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4a)

This compound was obtained as a white solid in 88% yield, with a melting point of 268–269 °C. The IR spectrum (KBr, cm^{-1}) showed characteristic bands at 3399 and 3308 (NH_2), 3016 (aromatic C–H), 2196 (C≡N), 1650 (C=O), and 1626 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.00 (dd, *J* = 6, 3 Hz, 1H, ArH), 7.79–7.74 (m, 1H, ArH), 7.54–7.46 (m, 2H, ArH), 7.33–7.14 (m, 6H, ArH and NH_2), 4.91 (s, 1H, CH), 2.32 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 169.04 (C=O), 159.70 (C-2), 155.01, 150.55, 141.47, 138.72, 134.93, 133.74, 129.30, 128.77, 125.88, 125.76, 125.59, 123.60, 119.85 (CN), 118.71, 56.26 (C-3), 41.47 (C-4), 21.48 (CH_3). Elemental analysis calculated for $C_{20}H_{14}N_2O_3$: C, 72.72%; H, 4.27%; N, 8.48%. Found: C, 72.57%; H, 4.08%; N, 8.11%.

2-Amino-4-(2-methoxyphenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4b)

This compound was obtained as a cream-colored powder in 83% yield, with a melting point of 266.5–267 °C. The IR spectrum (KBr, cm^{-1}) showed characteristic bands at 3389 and 3327 (NH_2), 3021 (aromatic C–H), 2187 (C≡N), 1667 (C=O), and 1648 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.13 (dd, *J* = 6, 3 Hz, 1H, ArH), 7.79–7.73 (m, 1H, ArH), 7.53–7.47 (m, 2H, ArH), 7.36–7.30 (m, 1H, ArH), 7.26–7.23 (m, 1H, ArH), 7.16 (s, 2H, NH_2), 7.09 (d, *J* = 6 Hz, 1H, ArH), 6.98 (t, *J* = 6 Hz, 1H, ArH), 5.19 (s, 1H, CH), 3.80 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 168.89 (C=O), 160.17 (C-2), 157.59, 154.96, 150.92, 134.82, 134.30, 129.89, 129.81, 129.05, 125.82, 125.75, 123.66, 121.46, 119.91 (CN), 118.72, 112.47, 56.35 (C-3), 55.54 (OCH_3), 36.48 (C-4). Elemental analysis calculated for $C_{20}H_{14}N_2O_4$: C, 69.36%; H, 4.07%; N, 8.09%. Found: C, 69.09%; H, 3.93%; N, 7.79%.

2-Amino-4-(3-chlorophenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4c)

This compound was obtained as a white powder with a yield of 91%, and a melting point of 244–244.5 °C. The IR spectrum (KBr, cm^{-1}) displayed prominent bands at 3396 and 3308 (NH_2), 3071 (aromatic C–H), 2197 (C≡N), 1648 (C=O), and 1627 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.12 (dd, *J* = 6, 3 Hz, 1H, ArH), 7.80–7.74 (m, 1H, ArH), 7.75–7.35 (m, 8H, NH_2, ArH), 5.05 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 169.08 (C=O), 159.81 (C-2), 157.04, 155.02, 149.53, 143.78, 134.99, 134.00, 131.37, 128.47, 128.32, 127.30, 125.92, 125.77, 123.63, 119.67 (CN), 118.69, 55.63 (C-3), 40.99 (C-4). Elemental analysis calculated for $C_{19}H_{11}ClN_2O_3$: C, 65.06%; H, 3.16%; N, 7.99%. Found: C, 64.79%; H, 3.02%; N, 7.77%.

2-Amino-4-(2-bromophenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4d)

This compound was obtained as a white powder with a yield of 86%, and a melting point ranging from 281–282 °C. The IR spectrum (KBr, cm^{-1}) displayed absorption bands at 3394 and 3331 (NH_2), 3046 (aromatic C-H), 2184 (C≡N), 1653 (C=O), and 1626 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.11 (dd, J = 6, 3 Hz, 1H, ArH), 7.77–7.68 (m, 2H, ArH), 7.50–7.26 (m, 7H, NH_2, ArH), 5.45 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 168.95 (C=O), 159.94 (C-2), 154.91, 149.47, 139.85, 134.98, 134.23, 133.74, 131.68, 130.52, 129.23, 125.92, 125.76, 123.61, 123.49, 119.42 (CN), 118.66, 55.18 (C-3), 41.44 (C-4). Elemental analysis calculated for $C_{19}H_{11}BrN_2O_3$: C, 57.74%; H, 2.81%; N, 7.09%. Found: C, 57.46%; H, 2.57%; N, 6.80%.

2-Amino-4-(4-fluorophenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4e)

The compound was obtained as a white powder with an 83% yield, and its melting point was observed between 228 and 229 °C. The IR spectrum (KBr, cm^{-1}) exhibited the following characteristic peaks: 3378 and 3304 (NH_2), 3068 (aromatic C-H), 2201 (C≡N), 1654 (C=O), and 1602 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.11 (d, *J* = 6 Hz, 1H, ArH), 7.76 (t, *J* = 6 Hz, 1H, ArH), 7.53–7.44 (m, 4H, ArH), 7.33–7.22 (m, 4H, NH_2, ArH), 5.03 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 169.04 (C=O), 163.77 (C-2), 160.54, 159.74, 154.99, 150.09, 137.62, 137.58, 134.93, 133.75, 130.55, 130.44, 125.87, 125.76, 123.59, 119.77 (CN), 118.65, 116.37, 116.09, 55.99 (C-3), 40.67 (C-4). Elemental analysis calculated for $C_{19}H_{11}FN_2O_3$: C, 68.26%; H, 3.32%; N, 8.38%. Found: C, 68.01%; H, 3.29%; N, 8.11%.

2-Amino-8-chloro-4-(3-methoxyphenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4f)

The compound was obtained as a white powder with an 88% yield and a melting point of 242–243 °C. The IR spectrum (KBr, cm^{-1}) exhibited prominent peaks at: 3395 and 3313 (NH_2), 3061 (aromatic C-H), 2197 (C≡N), 1645 (C=O), and 1629 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.01 (d, *J* = 6 Hz, 1H, ArH), 7.76 (dd, *J* = 6 Hz, 3 Hz, 1H, ArH), 7.58 (d, *J* = 6 Hz, 1H, ArH), 7.33 (t, *J* = 6 Hz, 3H, NH_2, ArH), 6.95-6.89 (m, 3H, ArH), 4.93 (s, 1H, CH), 3.77 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 168.00 (C=O), 160.04, 159.70 (C-2), 153.54, 150.64, 142.75, 134.70, 133.84, 130.59, 130.39, 124.80, 124.64, 121.15, 120.49, 119.73 (C≡N), 114.47, 113.48, 55.97, 55.58 (C-3), 41.39 (C-4). Elemental analysis calculated for $C_{20}H_{13}ClN_2O_4$: C, 63.09%; H, 3.44%; N, 7.36%. Found: C, 62.89%; H, 3.12%; N, 7.19%.

2-Amino-8-chloro-4-(2-chlorophenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4g)

The compound was isolated as a cream powder with a yield of 92% and a melting point of 262–262.5 °C. The IR

spectrum (KBr, cm^{-1}) displayed the following significant bands: 3389 and 3335 (NH_2), 3075 (aromatic C-H), 2187 (C≡N), 1654 (C=O), and 1633 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.01 (d, J = 3 Hz, 1H, ArH), 7.73 (dd, J = 6 Hz, 3 Hz, 1H, ArH), 7.54-7.35 (m, 7H, NH_2, ArH), 5.43 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 167.88 (C=O), 159.90 (C-2), 153.46, 149.78, 137.95, 134.77, 134.37, 133.15, 131.57, 130.49, 130.32, 128.63, 124.76, 124.63, 121.11, 119.37 (C≡N), 54.94 (C-3), 40.82 (C-4). Elemental analysis calculated for $C_{19}H_{10}Cl_2N_2O_3$: C, 59.24%; H, 2.62%; N, 7.27%. Found: C, 59.00%; H, 2.39%; N, 7.07%.

2-Amino-4-(3-bromophenyl)-8-chloro-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4h)

The compound was obtained as a cream powder with a yield of 84%, and it melted at 242.5–244 °C. The IR spectrum (KBr, cm^{-1}) showed the following key bands: 3389, 3312 (NH_2), 3057 (aromatic C-H), 2196 (C≡N), 1650 (C=O), and 1632 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.01 (d, J = 3 Hz, 1H, ArH), 7.76 (dd, J = 6 Hz, 3 Hz, 1H, ArH), 7.64-7.53 (m, 3H, NH_2, ArH), 7.44-7.36 (m, 4H, ArH), 5.05 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 168.05 (C=O), 159.71 (C-2), 153.57, 149.83, 143.83, 134.72, 134.07, 131.63, 131.42, 131.20, 130.41, 127.76, 124.86, 124.64, 122.65, 121.15, 119.59 (C≡N), 55.62 (C-3), 40.93 (C-4). Elemental analysis calculated for $C_{19}H_{10}BrClN_2O_3$: C, 53.11%; H, 2.35%; N, 6.52%. Found: C, 53.04%; H, 2.11%; N, 6.54%.

2-Amino-8-chloro-4-(4-fluorophenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4i)

The compound was obtained as a white powder with a yield of 82%, and it melted at 253–255 °C. The IR spectrum (KBr, cm^{-1}) exhibited the following prominent bands: 3378, 3306 (NH_2), 3071 (aromatic C-H), 2200 (C≡N), 1655 (C=O), and 1603 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.02 (d, J = 3 Hz, 1H, ArH), 7.77 (dd, J = 6 Hz, 3 Hz, 1H, ArH), 7.59 (d, J = 6 Hz, 1H, ArH), 7.48-7.43 (m, 2H, ArH), 7.35 (s, 2H, NH_2), 7.28-7.21 (m, 2H, ArH), 5.02 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 168.03 (C=O), 163.80, 160.57 (C-2), 159.64, 153.56, 150.44, 137.42, 137.38, 134.74, 133.86, 130.60, 130.49, 130.41, 124.80, 124.64, 121.14, 119.66 (C≡N), 116.38, 116.10, 55.95 (C-3), 40.62 (C-4). Elemental analysis calculated for $C_{19}H_{10}ClFN_2O_3$: C, 61.89%; H, 2.73%; N, 7.60%. Found: C, 61.66%; H, 2.56%; N, 7.39%.

2-Amino-8-chloro-4-(4-nitrophenyl)-10-oxo-4,10-dihydropyrano[3,2-*b*]chromene-3-carbonitrile (4j)

The compound was obtained as a cream powder with a yield of 81%, and it melted at 250–251.5 °C. The IR spectrum (KBr, cm^{-1}) showed prominent bands at 3370, 3321 (NH_2), 3071 (aromatic C-H), 2206 (C≡N), 1659 (C=O), 1602 (C=C), 1517, and 1351 (NO_2). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 8.27 (d, J = 6 Hz, 2H, ArH), 8.02 (d, J = 3 Hz, 1H, ArH), 7.79-7.72 (m, 3H, ArH), 7.58 (d, 1H, ArH), 7.48 (s, 2H, NH_2), 5.26 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 168.05 (C=O), 159.79 (C-2), 153.59, 149.36, 148.29, 147.66, 134.81, 134.21, 130.49, 130.03, 124.83, 124.65, 124.60, 121.14, 119.44 (C≡N), 55.08 (C-3), 41.00 (C-4). Elemental analysis calculated for $C_{19}H_{10}ClN_3O_5$: C, 57.66%; H, 2.55%; N, 10.62%. Found: C, 57.31%; H, 2.19%; N, 10.43%.

3. 1. *In vitro* Cytotoxicity Assessment of Synthesized Chromene Derivatives

The toxicity of the chromene compounds was evaluated using the MTT assay on MCF-7 (breast cancer) and 3T3 (normal) cell lines. Approximately all of the synthesized compounds displayed high levels of toxicity, resulting in IC_{50} values below 50 μg/mL for all compounds, except in three cases. The selectivity index of chromene compounds on cancerous and non-cancerous cell lines were calculated and reported in Table. 1. Among the tested compounds, **4f** and **4h** exhibited the highest toxicity. Compound **4f**, which had an IC_{50} of approximately 4 μg/mL in MCF-7 cells and an IC_{50} of nearly 20 μg/mL in normal 3T3 cells, had the highest cytotoxic effect among these samples. Additionally,

Table. 1 Summary of molecular features, IC_{50} values, and selectivity index of synthesized compounds in 3T3 and MCF-7 cells.

Compounds	Chemical Formula	Molecular Weight	3T3 (μg/ml)	3T3 (μM)	MCF-7 (μg/ml)	MCF-7 (μM)	Selectivity Index (SI)
4a	$C_{20}H_{14}N_2O_3$	330.34	>125	–	>125	–	–
4b	$C_{20}H_{14}N_2O_4$	346.34	>125	–	>125	–	–
4c	$C_{19}H_{11}ClN_2O_3$	350.76	48.40	137.98	79	225.22	0.612
4d	$C_{19}H_{11}BrN_2O_3$	395.21	>125	–	>125	–	–
4e	$C_{19}H_{11}FN_2O_3$	334.31	47	140.58	125.20	374.50	0.375
4f	$C_{20}H_{13}ClN_2O_4$	380.78	18.170	47.71	4.740	12.44	3.83
4g	$C_{19}H_{10}Cl_2N_2O_3$	385.20	32.450	84.24	42.000	109.03	0.772
4h	$C_{19}H_{10}BrClN_2O_3$	429.65	61.550	143.25	21.970	51.13	2.80
4i	$C_{19}H_{10}ClFN_2O_3$	368.75	32.780	88.89	30.870	83.71	1.061
4j	$C_{19}H_{10}ClN_3O_5$	395.76	34.123	86.22	43.800	110.67	0.779

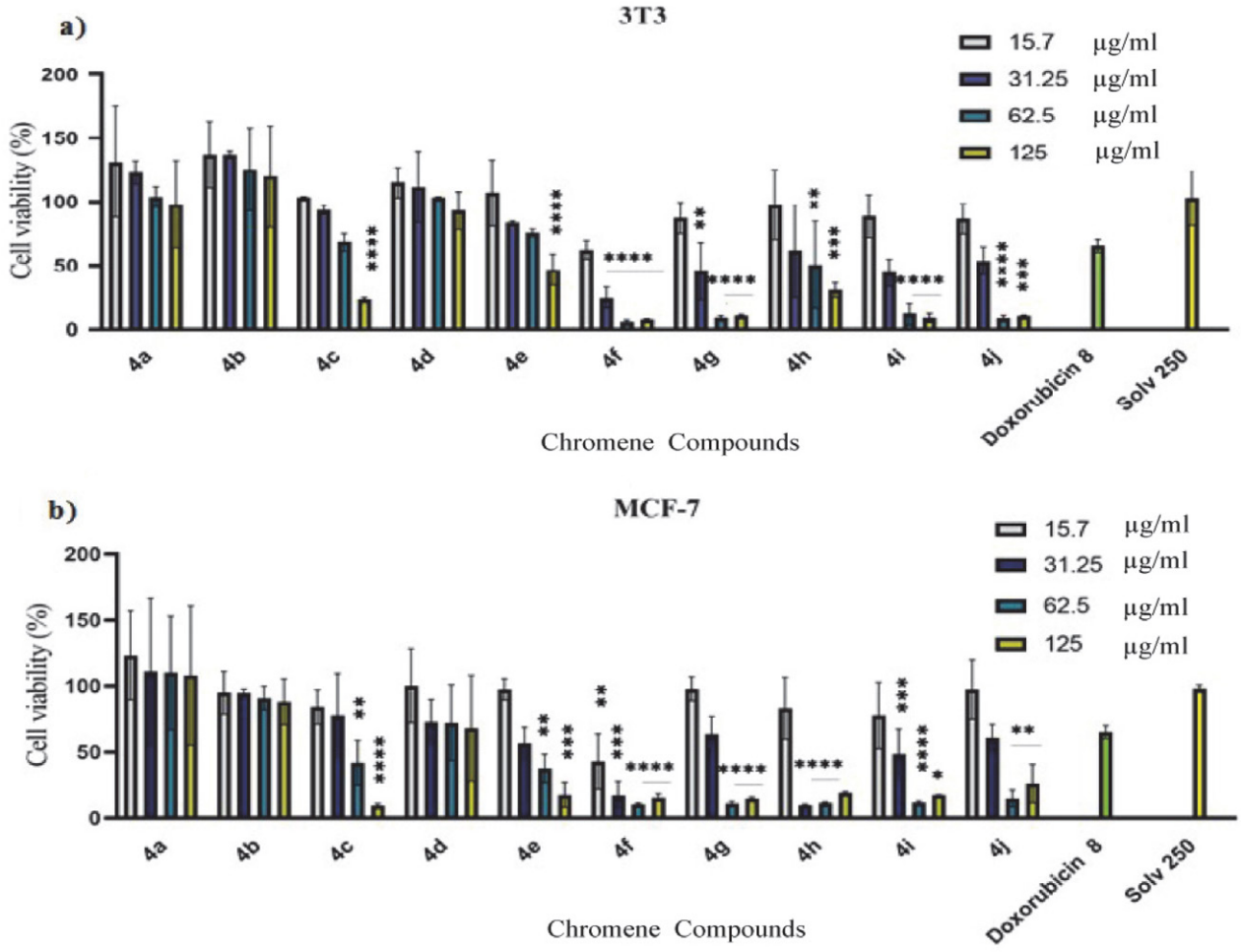


Figure 1. (a, b) The cytotoxic effects of chromene compounds at concentrations of 15.7–125 µg/mL were evaluated on 3T3 and MCF-7 cells after 24 hours of exposure. Doxorubicin (8 µg/mL) was used as a positive control. *Solv 250* refers to the solvent control containing 0.25% DMSO used to dissolve the test compounds. Data are expressed as mean ± SD (n = 3). Statistical significance was assessed by one-way ANOVA with Tukey's post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, **P < 0.0001).

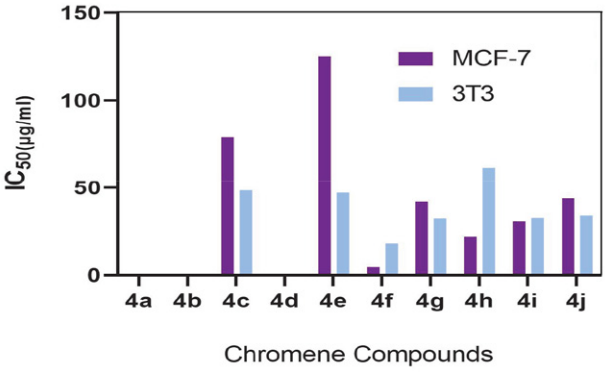


Figure 2. Bar chart showing IC_{50} values (µg/mL) of synthesized chromene derivatives against MCF-7 and 3T3 cell lines. Values were calculated from mean viability data (n = 3); error bars are not shown as IC_{50}s were derived from averaged curves. Corresponding numerical data are listed in Table 1

compound **4h** has an acceptable level of cytotoxicity, with an IC_{50} of about 21 µg/mL in MCF-7 cells and 61 µg/mL in 3T3 cells (Fig. 2). Interestingly, *in vitro* potential of chromene compounds was promising with selectivity index of 3.83 and 2.80 for **4f** and **4h**, respectively. These results highlight the potential of chromene derivatives as selective anticancer agents.

3. 2. Flow Cytometric Analysis of Apoptosis Using Annexin V/PI Staining

To further elucidate the cytotoxic mechanisms, flow cytometry analysis was performed on MCF-7 cells treated with compounds **4f** and **4h**. Flow cytometry analysis revealed that treatment with compound **4h** resulted in 13.57% viable cells, 6.2% early apoptotic cells, and 76% late apoptotic cells. For compound **4f**, 44.53% of the cells remained viable, while 4.04% and 25.83% underwent early and late apoptosis, respectively. Figure 4 depicts the morphology of MCF-7 cells treated with compounds **4f** and **4h**, which corresponds to the findings from the MTT and flow cytometry assays. These findings confirm that the cytotoxicity of these compounds is primarily mediated via induction of apopto-

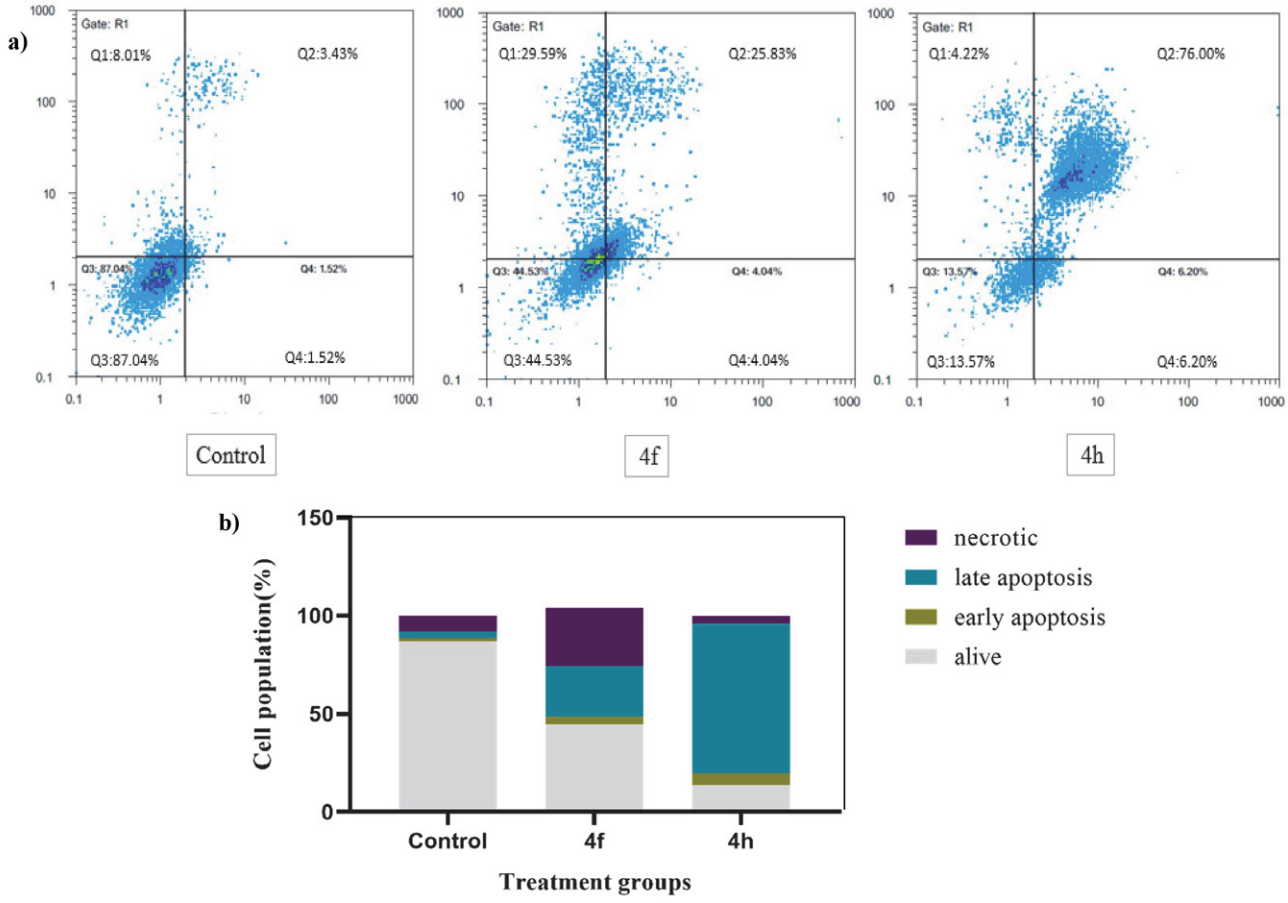


Figure 3. (a) Dot-plot flow cytometry histograms showing the distribution of MCF-7 cell populations after treatment with compounds **4f** (5 μg/mL) and **4h** (25 μg/mL) for 24 h. Cells were stained with Annexin V-FITC and propidium iodide (PI). The quadrants represent: viable cells (lower left, Annexin V– / PI–), early apoptotic (lower right, Annexin V+ / PI–), late apoptotic (upper right, Annexin V+ / PI+), and necrotic cells (upper left, Annexin V– / PI+). (b) Bar chart showing the percentage distribution of viable, early apoptotic, late apoptotic, and necrotic cells in each treatment group.

sis, consistent with previous studies on chromene derivatives demonstrating their ability to induce programmed cell death through mitochondrial pathways and regulation of apoptosis-related genes such as Bax, caspase-3, and Bcl-2.[31,34,38–40]

3. 3. Biological Significance of Chromene Derivatives

Chromenes are a class of naturally occurring oxygen-containing heterocycles, known for their simple

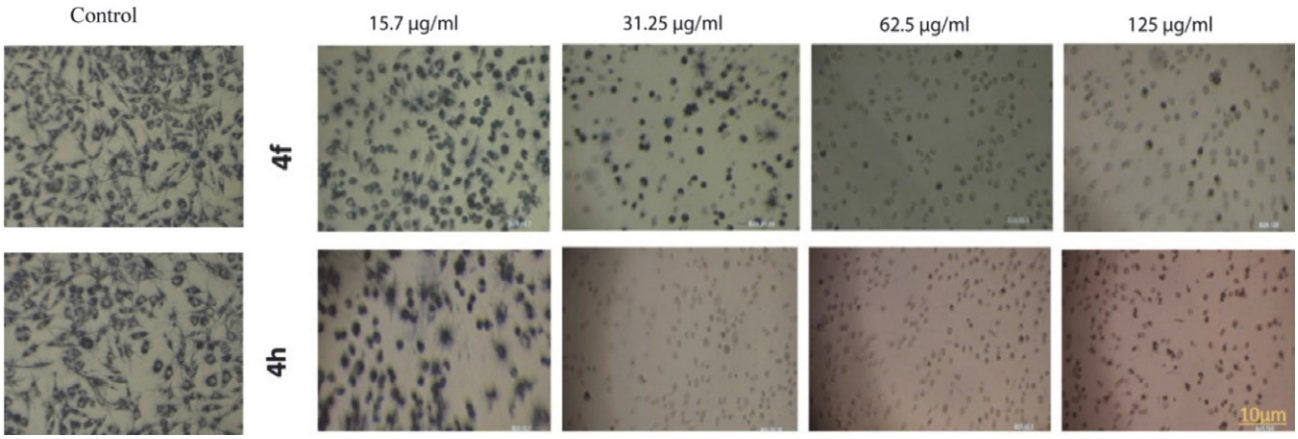


Figure 4. Phase-contrast images showing morphological changes in MCF-7 cells after 24-hour treatment with compounds **4f** and **4h** at increasing concentrations (15.7, 31.25, 62.5, and 125 μg/mL, left to right. Images were captured using phase-contrast microscopy at 40× magnification. Control: untreated MCF-7 cells. Cell shrinkage and death were more pronounced at higher concentrations, consistent with MTT and apoptosis assay results.

structure and minimal adverse effects, making them attractive for drug development.[41,42] They form the core of many flavonoids and possess a broad range of biological activities including anticancer, antimicrobial, neuroprotective, and anti-HIV effects.[41–47] Several chromene derivatives have shown significant cytotoxicity by binding to the colchicine-binding site of tubulin, inhibiting polymerization, and causing cell cycle arrest leading to apoptosis.[23,24] Gourdeau et al. demonstrated the anti-vascular and anticancer properties of certain chromene derivatives, highlighting their potential in clinical cancer therapy, particularly due to their anti-angiogenic effects and synergy with chemotherapeutic agents.[24]

3. 4. Structure–activity Relationship Analysis of Chromene Derivatives Based on Cytotoxicity Results

Structure–activity relationship (SAR) analysis revealed that substitution patterns on the chromene core significantly influence activity. In particular, compounds **4f** and **4h** contain *meta*-positioned substituents on the 4-aryl phenyl ring, a methoxy (-OCH_3) group in **4f** and a bromine (-Br) atom in **4h**, which may explain their higher potency.[48] Previous studies have also noted that electron-donating groups at the 7th position of the chromene scaffold enhance pharmacological effects, while electron-withdrawing groups tend to reduce activity.[48] Zhang et al. reported anticancer effects of chromene derivatives via modulation of the p53 pathway and downregulation of VEGF, ICAM1, and MMP-2, supporting their antitumor potential.[49]

4. Conclusion

In summary, we successfully synthesized and characterized a series of chromene-based derivatives via a one-pot, three-component reaction. The structures of the synthesized compounds were confirmed by spectroscopic methods, including IR and NMR (^{1}H and ^{13}C NMR), and elemental analysis. Biological evaluations revealed that several of these compounds, particularly **4f** and **4h**, exhibit potent cytotoxic effects against MCF-7 breast cancer cells, with IC_{50} values of 4.74 μg/mL and 21.97 μg/mL, and favorable selectivity indexes of 3.83 and 2.80, respectively. Flow cytometry analysis confirmed their ability to induce apoptosis, further supporting their anticancer potential. These findings underscore the value of chromene scaffolds as promising leads for breast cancer therapy.

Nevertheless, to fully elucidate their therapeutic potential, further research is warranted, including evaluations across diverse cancer cell lines, *in vivo* toxicity and efficacy assessments, and deeper mechanistic investigations such as gene expression profiling and protease interaction studies. Such efforts may lead to the development of more effective chromene-based agents for overcoming chemo-resistance in cancer therapy.

Declarations

Data availability statement

The original contributions made in this study are part of the article/supplementary material. For additional questions, please contact the corresponding author.

Author contributions

BB, SS, BA and MA designed and supervised the project, supplied all reagents; and edited the manuscript. YA, BB, and MA wrote the original draft and collaborated with AAK, AA, and FH to carry out the experiments and analysis of the results. All authors participated in the interpretation, and analysis of the data and review of the manuscript.

Conflict of interest

The authors confirm that the research was carried out without any commercial or financial relationships that could be perceived as a potential conflict of interest.

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Ethics approval and consent to participate

The study did not involve human participants or animals.

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Povzetek

Rak ostaja vodilni vzrok smrti po vsem svetu, zato so potrebni novi terapevtski pristopi. V tej študiji so avtorji sintetizirali in karakterizirali derivate 2-amino-3-karbonitril kromena in ocenili njihove citotoksične učinke na celične linije 3T3 in MCF-7. Karakterizacija je vključevala tališče, IR, NMR in elementno analizo. Citotoksičnost je bila ocenjena z MTT testom, pri čemer so bile izračunane vrednosti IC_{50}, indukcija apoptoze pa je bila potrjena s pretočno citometrijo z barvanjem z anexinom V/propidijevim jodidom. Spojine **4f** in **4h** so pokazale znatno citotoksičnost proti celicam raka dojke, z vrednostmi IC_{50} 4,74 in 21,97 μg/ml ter indeksi selektivnosti 3,83 in 2,80. Povečana populacija apoptičnih celic podpira njihov proapoptični potencial. Ti rezultati kažejo, da derivati kromena, sintetizirani z enostopenjsko metodo, predstavljajo obetavne kandidate za nadaljnji razvoj zdravil proti raku.