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4-methyl-coumarins with cytotoxic activity against T24 and RT4 human bladder cancer cell lines

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Bladder cancer is one of the most prevalent malignancies of the genitourinary tract, and approximately 25% of patients with superficial cancers develop invasive and metastatic pathology. Coumarins and their derivatives have antiproliferative activity and induce apoptosis in several cancer cell lines. Due to the potential therapeutic applications of these compounds, a series of 4-methylcoumarins was synthesized to investigate the antitumor cytotoxic effect in the T24 and RT4 human bladder cancer cell lines. The microwave-assisted synthesis of the coumarins via Pechmann condensation with modifications at position 7 was performed with excellent yields (74-100%). The 7,8-dihydroxy-4-methyl-2-oxo-2H-chromene-5-carboxylic acid derivative (3c) exhibited greater cytotoxicity against T24 cells, which represent a more malignant cell line compared to RT4. In the T24 cells, the cell cycle analysis revealed a large number of cells in the sub-G1 phase after treatment with 3c, which is indicative of apoptosis. The apoptotic death was confirmed by anexinn-V staining assay. Based on the chemical structures of the compounds, it is possible to suggest a positive influence of the 5-carboxycoumarin ring group on the cytotoxic activity. These results indicate that these new compounds are promising for further chemical modulation to discover a new antitumor agent.

Introduction

Bladder cancer is the seventh most common cancer worldwide and is one of the most prevalent genitourinary malignancies (1,2). Approximately 30% of bladder cancers are classified as muscle-invasive tumors and are associated with a significant risk of subsequent metastases, and only 35% of patients achieve a survival rate of 10 years, demonstrating the aggressive nature of this disease (3-4). The intravesical administration of Bacillus Calmette-Guerin (BCG) after transurethral resection is the most effective treatment of superficial bladder cancer. Nevertheless, side effects of the BCG therapy are common, and approximately one third of patients do not respond to this treatment (5-7). Considering the ineffectiveness of various therapeutic strategies used in bladder cancer treatment (8), the search for effective and selective molecules to target this pathology is essential.

Several classes of potential antitumor agents have been identified, such as coumarins. One antitumor coumarin is warfarin, which is used in chemotherapy cell lines V2 of lymphocytes, macrophages and granulocytosis. Umbelliferone is another coumarin that has been used in a lung cancer cell line (9). Coumarins (2*H*-chromen-2-ones) can be considered privileged structures due to their wide range of biological activities, such as antitumor (10-12), antiviral (13), antioxidant (14) and antibacterial (15) activities. Coumarin and its derivatives cause significant changes in the regulation of the immune response, cell growth and differentiation. Various mechanisms have been proposed including the

inhibition of protein kinase, phosphodiesterase and the negative regulation of expression of oncogenes (16-17).

In a previous study, we identified a natural coumarin, 5-methoxy-6,7-methylenedioxycoumarin, which was isolated from the *Pterocaulon* species. This coumarin exhibited significant cytotoxicity against glioma cells. This oxygenated compound exhibited IC50 values of 34.6 μM and 31.6 μM against human (U138-MG) and rat (C6) glioma cells, respectively. In addition, the cytotoxic effect induced by this coumarin in glioma cells was not observed in organotypic cell cultures, indicating a selective cytotoxicity for tumor cells (18). These results encouraged us to proceed with the synthesis of coumarins and evaluation of their antitumor activity to determine a prototype for further studies of the structure activity relationship. In addition, we demonstrated that 4-methycoumarins has a potent inhibitory effect on the human bladder cancer cell lines.

Various methods can be employed for coumarin synthesis, and the chosen method is based on the desired substitution pattern around the nucleus. Some methodologies are based on the Perkin (19), Knoevenagel (20-22), Reformatsky (23), Wittig (24-25) and Pechmann reactions (26). The Pechmann condensation is the most straightforward method for synthesizing coumarins. This reaction involves the condensation of β-ketoesters and phenolic compounds in acidic media (**Scheme 1**) via a transesterification/Michael addition mechanism (27-28). The application of microwave (MW) irradiation has been reported to be a powerful tool for synthesis of 4-substituted coumarins (29). However, no report has been published on 7-*O*-substituted coumarins. The application of MW irradiation in organic synthesis offers significant advantages over conventional heating methods, such as substantial rate enhancements, cleaner formation of products and improved yields (30).

Results and Discussion

The synthesis of several 4-methyl-coumarin derivatives is shown in **Scheme 1**. The MW Pechmann reaction of resorcinol **1a** with ethyl acetoacetate **2** in the presence of catalytic amounts of concentrated HCl afforded 7-hydroxy-4-methyl-2*H*-chromen-2-one **3a** in 99% yield. The same conditions led to the formation of **3b** (80% yield) and **3c** (76% yield) from pyrogallol **1b** and gallic acid **1c**, respectively.

To explore the influence of the substituent at the 7-position of the coumarin in the screening of the cytotoxic activity against human bladder cancer cell lines, **3a** was reacted with a variety of electrophiles to yield 7-*O*-substituted coumarins **4-7** (**Scheme 2**). All of the reactions were carried out under MW irradiation for very short times (15-20 minutes) to yield compounds with high purity and in excellent yields (74-99%).

Initially, the antitumor effects of all of the 4-methylcoumarin molecules synthesized were evaluated in the T24 cell line in a concentration range of 5 to 100 μM. With the exception of **3c**, the screening of 4-methylcoumarin indicated weak activity in the T24 cells after 24 h of treatment (Fig. 1). In this paper, coumarins alkylated at 7-OH with allyl **4**, ethyl acetate **5**, tosyl **6** and benzyl **7** groups were devoid of cytotoxic activity similar to their corresponding non-alkylated coumarin, **3a**. These results indicate that position 7 of the coumarin ring may not exert significant effect on the cytotoxic activity against bladder cancer cell line. In addition, the addition of a hydroxyl group at position 8 of the coumarin ring had no effect on the antiproliferative activity (based on **3b** and **3c, Scheme 1.**).

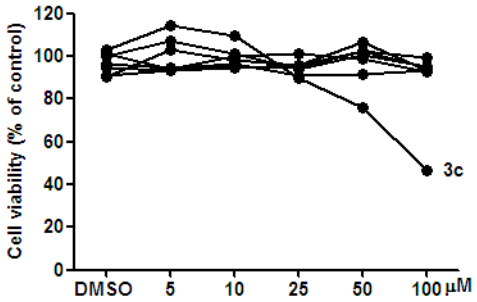


Fig 1. Effect of **3a**, **3b**, **3c**, **4**, **5**, **6** and **7** on the cell viability in the T24 human bladder cancer cell line. The cells were grown on a 96-well plate and, after reaching semi-confluence, the cells were treated with the derived coumarins or DMSO (vehicle group). After 24 h of treatment with concentrations of 5, 10, 25, 50 and 100 μM, the cell viability was evaluated using a MTT assay, as described in the Materials and Methods section. The cell viability was represented in relationship to DMSO. The data represent the means of at least three independent experiments performed in triplicate ± SD. The data were analyzed for statistical significance by one-way ANOVA followed by a Tukey post-hoc analysis.

As observed in **Fig. 1**, among all of the molecules tested, the best result was obtained with **3c**, which exhibited excellent cytotoxic activity against the T24 bladder cancer cell line and significantly reduced the cell viability (21.37%) at a concentration of 10 μM after 48 h of treatment (**Fig. 2**). Due to the large reduction in

cell viability at concentrations of 25 µM (62.26%), 50 µM (63.95%) and 100 µM (65.64%) after 48 h of treatment (**Fig. 2**), we selected the same doses and a time of 24 h for use in further experiments.

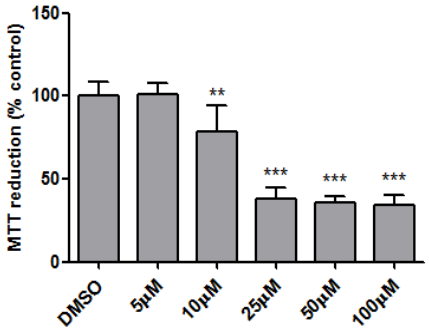
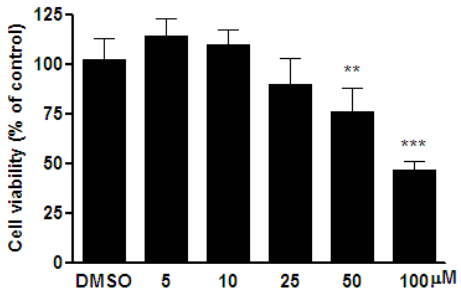
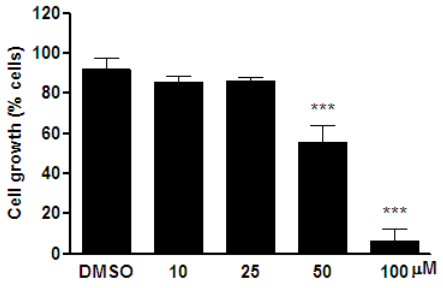


Fig. 2. Effect of **3c** on cell viability in the T24 human bladder cancer cell line. The cells were grown on a 96-well plate, and after reaching semi-confluence, these cells were treated with **3c** or DMSO (vehicle group). After 48 h of treatment with concentrations of 5, 10, 25, 50 and 100 µM, the cell viability was evaluated using a MTT assay, as described in the Materials and Methods section. The cell viability is represented in relationship to DMSO. The data represent the mean of at least three independent experiments performed in triplicate ± SD. The data were analyzed for statistical significance by a one-way ANOVA followed by Tukey post-hoc analysis. **Significantly different from DMSO ($p<0.01$). ***Significantly different from DMSO ($p<0.001$).

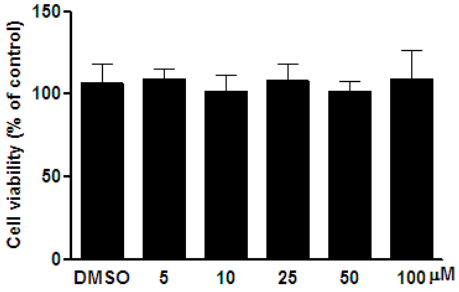
When the T24 cells was exposed to different concentrations for 24 h, **3c** demonstrated promising results and provided a significant reduction in the cell viability (24.14%) (**Fig. 3A**) and in the cell count (44.52%) with a 50 µM treatment (**Fig. 3B**). By comparing molecules **3b** and **3c**, the addition of an acid group at position C5 increased the cytotoxic activity in this cell line.



(A)



(B)



(C)

Fig. 3. Effect of **3c** on the cell viability in the T24 human bladder cancer cell line (**A**). Cell count assay in the T24 human bladder cancer cell line (**B**) and cell viability in RT4 human bladder cancer cell line (**C**). The cells were grown on a 96-well plate, and after reaching semi-confluence, these cells were treated with **3c** or DMSO (vehicle group) with concentrations of 5, 10, 25, 50 and 100 µM to evaluate cell viability or with 10, 25, 50 and 100 µM to evaluate the cell count assay. After 24 h of treatment, the cell viability was evaluated using a MTT assay, and the cell count assay was performed in a hemocytometer, as described in the Materials and Methods section. The cell viability and cell count are represented in relationship to DMSO. The data represent the mean of at least three independent experiments performed in triplicate ± SD. The data were analyzed for statistical significance by a one-way ANOVA followed by Tukey post-hoc analysis. **Significantly different from DMSO ($p<0.01$). ***Significantly different from DMSO ($p<0.001$).

Finally, **3c** was tested against the RT4 human bladder cancer cell line, which is representative of a superficial noninvasive cancer, and in the MTT experiment, this molecule did not exhibit activity (**Fig. 1**). The highest toxicity in the T24 cell line was derived from an

invasive bladder tumor with metastatic potential, and this cell line is more sensitive to treatment with **3c** compared to the RT4 cell line, which is derived from a noninvasive cancer. The T24 strain is able to divide faster compared to the RT4 strain. Therefore, drug (**3c**) metabolism is accelerated. This accelerated metabolism explains the effect of chemotherapy of tumor cells as well as the adverse effects on proliferating cells, such as skin and hair cells. A major genetic difference between the established RT4 and T24 cell lines is the status of p 53 and PTEN expression. Although RT4 cells are wild-type for p 53 and PTEN, the T24 cells are null for p 53 and PTEN (31).

To investigate the mechanism by which **3c** decreases T24 cell viability along with the associated arrest in the cell cycle progression, flow cytometry analysis was performed on cells treated with 25, 50 and 100 μM **3c** for 24 h. The results of the cell cycle analysis revealed a larger cell accumulation in the Sub-G1 phase after treatment with 50 and 100 μM of **3c**, which may be characteristic of apoptotic death (**Fig. 4**). Previous studies reported similar results where coumarins induced apoptosis by increasing the percentage of cells in the Sub-G1 phase in human cervical cancer HeLa and human lung adenocarcinoma (32).

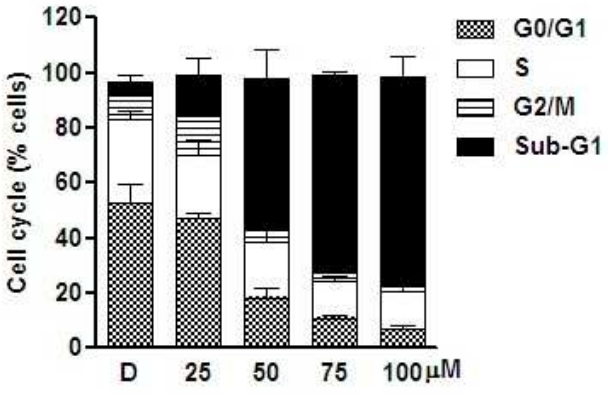


Fig. 4. Effect of **3c** on cell cycle distribution of the T24 human bladder cancer cell line. The T24 cells were treated with DMSO (vehicle group) or **3c** (25, 50 and 100 μM) for 24 h, and its content was analyzed by flow cytometry, as described in the Materials and Methods section. The reported values are the relative number of cells in the sub-G1, G1, S and G2/M phases of the cell cycle. The data were obtained from three independent experiments.

Because **3c** causes an increase in the cells in the Sub-G1 phase, possible induction of apoptosis was analyzed using *Annexin* V/Propidium Iodide (PI) double staining by flow cytometry. Therefore, we decided to investigate if the same time and concentration used to analyze the cell cycle caused necrosis, apoptosis or both in T24 cells. As shown in **Fig. 5**, the percentage of apoptosis in T24 cells increased significantly after treatment with 50 μM (22.57%) of **3c** compared to DMSO. Apoptosis, which is one of the most fundamental biological processes in eukaryotes where individual cells die by activating intrinsic "suicide" mechanisms, may play a key role in damaging cancer cells by causing a variety of injuries (33). Previous studies have demonstrated that coumarins induce apoptotic cell death in cancer cell lines, such as lung carcinoma (34), which is in agreement with our results.

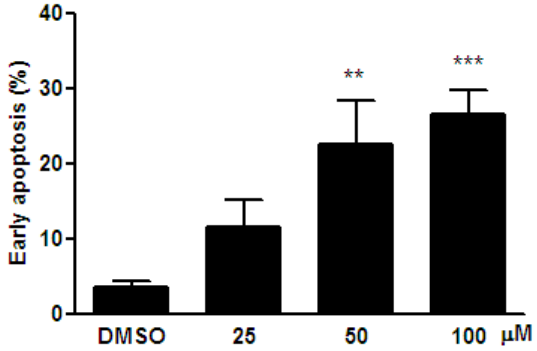


Fig. 5. Apoptotic effect of **3c** on the T24 human bladder cancer cell line. T24 cells were treated with DMSO (vehicle group) or **3c** (25, 50 and 100 μM). After 24 h of treatment, the cells were double-stained with *annexin V* and PI and analyzed by flow cytometry, as described in the Materials and Methods section. The graph shows the quantitative analysis of early apoptosis in cells treated with **3c**. The data were obtained from three independent experiments. * Significantly different from DMSO (p<0.05).

Although **3c** exerts various biological properties, further studies are needed to determine its exact mechanism of action on bladder cancer cells. Because antioxidants have different effects in cancer treatment (35), the antioxidant activity of **3c** was previously measured using different methods (i.e., the 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) method, cyclic voltammetry and the antioxidant capacity against peroxyl radicals (ACAP) method). Compound **3c** was confirmed to be the most active (36). In addition, because in vitro conditions are very different from an in vivo environment, an approved method for animal models is required (37) to evaluate 3c effects on biological systems.

Experimental

General

All of the chemicals were of research grade and used as obtained. The ^{1}H and ^{13}C NMR spectra were obtained using an INOVA-300 spectrophotometer with standard pulse sequences operating at 300 MHz for the ^{1}H NMR and 75 MHz for the ^{13}C NMR or at 400 and 100 MHz, respectively, using a Brucker Avance NMR spectrometer with DMSO-d_6 and $CDCl_3$, respectively, as the solvent. The chemical shifts are reported as σ values (ppm) relative to TMS (0.0 ppm), and the *J* values are reported in Hz. The NMR multiplicities br s, s, d, t, q, and m stand for broad singlet, singlet, doublet, triplet, quartet and multiplet, respectively. TLC analyses were performed on Merck® silica plates 60 GF 254. The melting points (m.p.) were determined on a System Kofler type WME apparatus and are uncorrected. The term room temperature means 20-30 °C. The products were identified by comparison of the melting points and spectroscopic data with previously reported values.

Maintenance of cell lines

The human bladder cancer cell lines (i.e., T24 (derived from an invasive bladder tumor with metastatic potential) and RT4 (representing a superficial noninvasive cancer)) were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). T24 was maintained in RPMI culture medium, and RT4 was maintained in DMEM culture medium. The cells were grown and maintained in flasks with culture medium containing 0.5 U/mL penicillin/streptomycin antibiotics and supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were maintained at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere consisting of 5% CO_2 in air.

Cell treatment

4-Methylcoumarin was dissolved in cell culture grade dimethyl sulfoxide (DMSO). The maximum final concentration of DMSO in the culture medium was 0.25% (v/v), and DMSO was used as the vehicle group in all of the experiments. The control cultures were prepared with the addition of culture medium or DMSO (vehicle control). A concentration of 0.25% had no effect on the cell culture and does not interfere with the experiments. The human bladder cancer cell lines were seeded according to each experiment, and after reaching semi-confluence, the cultures were exposed to 4-methylcoumarin (5, 10, 25, 50 and 100 μM) for 24 or 48 h.

Cell viability assay

The MTT method is based on the ability of metabolically active mitochondria from viable cells to reduce the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals. The bladder cancer cell lines were plated in a 96-well plate at 4×10^3 (T24) and 10×10^3 (RT4) per well. After reaching semi-confluence, the cultures were treated as described above. The control culture was prepared with the addition of culture medium (cell viability control) or DMSO (vehicle control) in the absence of treatment. After 24 and 48 h of treatment, the culture medium was removed, and the cells were washed twice with phosphate buffered saline (PBS) (pH 7.4). After removing the PBS, 90 μL of culture medium and 10 μL of MTT (5mg/mL) were added to each well. The cells were incubated for 3 h, and the solution was removed from the precipitate. A total of 100 μL of DMSO was added to the wells, and the optical absorbance was measured at 630 and 570 nm using an ELISA plate reader. This absorbance was linearly proportional to the number of live cells with active mitochondria. The results are expressed as a percentage of cell viability compared to that obtained for DMSO.

Cell counting

T24 cells were seeded at 6×10^3 cells per well in 24-well plates and allowed to grow until reaching semi-confluence. The cells were treated with 4-methylcoumarin as described above. After 24 h of treatment, the medium was removed. The cells were washed twice with CMF (calcium and magnesium free buffer), and 200 μL of a 0.05% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer. The results are expressed as the percentage of cells compared to that obtain with DMSO.

Cell cycle analysis

The T24 cell line was plated in a 6-well plate at 3.5×10^4 per well and incubated for three days to achieved adherence. After reaching semi-confluence, the cells were treated with 25, 50 and 100 μM of 7,8-dihydroxy-4-methyl-2-oxo-2*H*-chromene-5-carboxylic acid (**3c**) for 24 h. At the end of the treatment, the medium and cells were harvested and centrifuged at 400 x *g* for 6 min. The cells were suspended in PBS (pH 7.4) and counted. A quantity of 4×10^5 cells was added to a

solution containing 0.5 mM Tris-HCl (pH 7.6), 3.5 mM trisodium citrate, NP 40 0.1% (v/v), 100 µg/mL RNAse and 50 µg/mL PI. After 15 min, the data were collected using flow cytometry (FACS Calibur cytometry system, BD Bioscience, Mountain View, CA, USA). The obtained data were analyzed using the FLOWJO® software.

Annexin V/PI flow cytometric staining technique

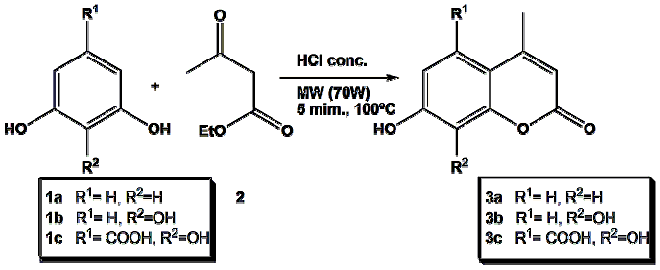
Apoptotic or necrotic cells were quantified using an *annexin V*-FITC-propidium iodide (PI) kit with double staining according to the instructions provided by the manufacturer (BD Pharmingen, Franklin Lakes, NJ, USA). The T24 cells seeded in the 6-well plates at 3.5×10^4 cells per well were maintained for three days to allow for adherence. After reaching semi-confluence, the cells were treated with 25, 50 and 100 µM of 7,8-dihydroxy-4-methyl-2-oxo-2*H*-chromene-5-carboxylic acid (**3c**) for 24 h. At the end of the treatment, the medium and cells were harvested and centrifuged at 400 x *g* for 6 min. The cells were suspended in cold PBS (pH 7.4) and counted. A concentration of 10^6 cells/mL was centrifuged and suspended in binding buffer containing FITC-conjugated *annexin* V and PI. The samples were incubated for 15 min in the dark at room temperature. The quantification of apoptotic and/or necrotic cells was screened by dual-color flow cytometry technique using a FACS Calibur cytometry system (FACS Calibur, BD Bioscience, Mountain View, CA, USA). The obtained data were analyzed using the FLOWJO® software. The cells were classified as follows: live cells (*Annexin*-/PI-); early apoptotic cells (*Annexin*+/PI-); late apoptotic cells (*Annexin*+/PI+); and necrotic cells (*Annexin*-/PI+).

Statistical analysis

All of the experiments were performed at least three times, and the results are expressed as a mean ± SD and analyzed by one-way ANOVA followed by Tukey post-hoc test using the GraphPad Prism Software. The differences were considered significant in relationship to DMSO when $p<0.05$.

B Synthetic procedures

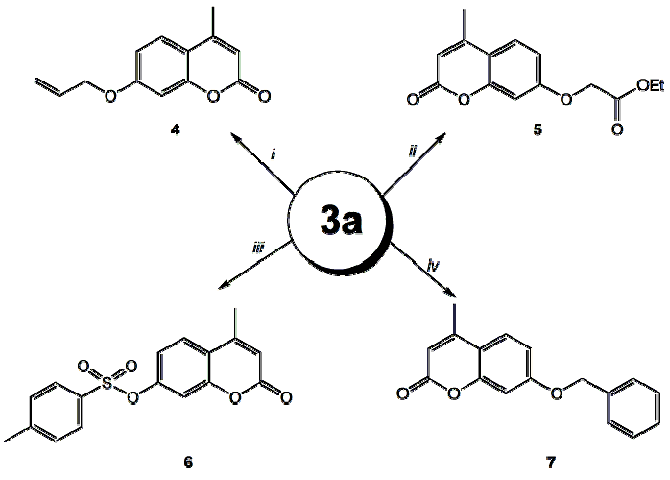
Microwave heating: The experiments were carried out in a CEM Discover® Microwave in closed vessels and subjected to MW irradiation at 70 W for the specified period shown in **Scheme 1** and **2**. All of the synthesized compounds were identified by comparison to literature data. The melting points and spectral data are in agreement with those previously reported.



Scheme 1. Pechmann condensation between ethyl acetoacetate and phenolic compounds under microwave irradiation.

*Synthesis of 2*H*-chromen-2-ones* (**Scheme 1**). A mixture of resorcinol, pyrogallol or gallic acid (2 equiv.), ethyl acetoacetate (1 equiv.) and concentrated HCl (0.1 equiv.) was placed in a quartz reaction vessel and allowed to react under microwave irradiation at a temperature of 100 °C for 5 min, as shown in **Scheme 1**. After total consumption of the ketoester, as indicated by TLC, the reaction mixture was cooled to room temperature, and cold water was added. The white precipitate that formed was filtered, washed with cold water and dried under vacuum.

*Synthesis of 7-*O*-substituted coumarins* (**Scheme 2**). 7-Hydroxy-4-methyl-2*H*-chromen-2-one (1 equiv.) was dissolved in MeCN. To this solution, a base (2 equiv.) and a electrophile (2 equiv.) were added at 70 °C for 15-20 min, as shown in **Scheme 2**. At the end of the reaction, which was monitored by TLC, MeCN was added followed by extraction with water and brine. The filtrate was dried over Na_2SO_4, stirred for 10-15 min filtrated and evaporated under vacuum. When necessary, the product was crystallized from diethyl ether or pentane.



Scheme 2. Reaction conditions: *i*) allyl bromide, K_2CO_3, MeCN, 70 W, 70 °C, 15 min, 81%; *ii*) ethyl 2-bromoacetate, K_2CO_3, MeCN, 70 W, 70 °C, 15 min, 85%; *iii*) tosyl chloride, Et_3N, MeCN, 70W, 70 °C, 20 min, 99%; iv) benzyl bromide, K_2CO_3, MeCN, 70 W, 70 °C, 20 min, 93%.

*7-hydroxy-4-methyl-2*H-*chromen-2-one* ***3a*** (38): m.p. = 187-189 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.37 (s, 3H, CH_3), 6.04 (s, 1H, CH), 6.76-6.84 (m, 2H, CH_{arom}), 7.43 (d, J = 8.6 Hz, 1H, CH_{arom}), 10.13 (br s, 1H, OH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.9, 102.1, 109.9, 111.7, 112.5, 125.1, 152.6, 154.3, 160.5, 160.9.

*7,8-dihydroxy-4-methyl-2*H-*chromen-2-one* ***3b*** (39): m.p. > 250 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.38 (s, 3H, CH_3), 6.06 (s, 1H, CH), 6.85 (d, J = 8.6 Hz, 1H, CH_{arom}), 7.02 (d, J = 8.6 Hz, 1H, CH_{arom}). ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.9, 108.8, 111.6, 112.7, 116.8, 133.7, 141.5, 148.2, 153.9, 161.7.

7,8-dihydroxy-4-methyl-2-oxo-2H-chromene-5-carboxylic acid ***3c*** (40): m.p. > 250 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 2.43 (s, 3H, CH_3), 6.87 (s, 1H, CH), 9.11 (s, 1H, CH_{arom}). ^{13}C NMR (75 MHz, DMSO-d_6) δ 20.2, 109.3, 111.3, 119.2, 120.9, 138.7, 146.7, 147.4, 148.2, 158.3, 168.0.

*7-(allyloxy)-4-methyl-2*H-*chromen-2-one* ***4*** (41): m.p. = 72-75 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.40 (s, 3H, CH_3), 4.61 (d, J = 3.8 Hz, 2H, CH_2), 5.33-5.36 (dd, J^{cis} = 10.4 Hz, J^{gem} = 1.3 Hz, 1H, CH_2), 5.42-5.48 (dd, J^{trans} = 17.1 Hz, J^{gem} = 1.4 Hz, 1H, CH_2), 5.99-6.12 (m, 1H, CH), 6.14 (s, 1H, CH), 6.83 (d, J = 2.4 Hz, 1H, CH_{arom}), 6.87-6.91 (dd, J^1 = 8.8 Hz, J^2 = 2.4 Hz, 1H, CH_{arom}), 7.50 (d, J = 8.8 Hz, 1H, CH_{arom}). ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.6, 69.2, 101.7, 111.9, 112.7, 113.6, 118.4, 125.5, 132.1, 152.5, 155.1, 161.3, 161.5.

Ethyl 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetate ***5*** (42-43): m.p. = 98-101 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.73 (t, J = 7.0 Hz, 3H, CH_3), 2.67 (s, 3H, CH_3), 4.32 (q, J = 7.1 Hz, 2H, CH_2), 5.38 (s, 2H, CH_2), 6.51 (s, 1H, CH), 7.18-7.79 (m, 3H, CH_{arom}). ^{13}C NMR (400 MHz, DMSO-d_6) δ 14.5, 18.6, 61.3, 65.4, 101.9, 111.9, 112.8, 114.1, 126.9, 153.7, 154.9, 160.5, 161.0, 168.6.

4-methyl-2-oxo-2H-chromen-7-yl-4-methylbenzenesulfonate ***6*** (43): m.p. > 250 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.33 (s, 3H, Ar-CH_3), 2.38 (s, 3H, CH_3), 6.18 (s, 1H, CH), 6.76 (s, 1H, CH_{arom}), 6.99 (d, J = 8.7 Hz, 1H, CH_{arom}), 7.26 (d, J = 8.2 Hz, 2H, CH_{arom}), 7.48 (d, J = 8.8 Hz, 1H, CH_{arom}), 7.64 (d, J = 8.2 Hz, 2H, CH_{arom}). ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.7, 21.7, 110.8, 115.0, 118.7, 118.8, 125.7, 128.4, 130.0, 131.8, 146.0, 151.5, 151.7, 153.8, 160.0.

7-(benzyloxy)-4-methyl-2H-chromen-2-one ***7*** (44): m.p. = 132-134 °C, ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 2.37 (s, 3H, CH_3), 5.21 (s, 2H, CH_2), 6.20 (s, 1H, CH), 7.00-7.06 (m, 2H, CH_{arom}), 7.34-7.49 (m, 5H, CH_{arom}), 7.67 (d, 1H, CH_{arom}). ^{13}C NMR (400 MHz, DMSO-d_6) δ 18.5, 70.3, 102.0, 111.7, 113.1, 113.7, 126.9, 128.3, 128.5, 128.9, 136.7, 153.8, 155.1, 160.53, 161.74.

Conclusions

In summary, among all the synthesized coumarins, **3c** exhibited the best cytotoxic potential against a bladder cancer cell line leading to a reduction in the viability of T24 cells. These results may indicate selectivity for malignant cells because there was a higher cytotoxicity against T24 cells, which represents a higher degree of malignancy compared to RT4. In addition, **3c** increased the cell number in the Sub-G1 phase inducing apoptosis, which was confirmed by *annexin V* staining in this cell line after 24 h of treatment. Therefore, the cytotoxic activity determined for 7,8-dihydroxy-4-methyl-2-oxo-2*H*-chromene-5-carboxylic acid, **3c,** is suggests the need for further testing *in vivo* in a bladder cancer animal model.

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