Research Article

Structure-activity relationship of anticancer potential of 4-hydroxycoumarin and its derivatives: A comparative study

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Abstract

Objective: In view of high degree of bioactivity shown by 4-hydroxycoumarin and the compounds derived from it, our main aim was to carry out the synthesis of various structurally varied derivatives of 4-hydroxycoumarin and study the effect on their anti-cancer potential. **Materials and methods:** The reaction of 4-hydroxycoumarin with aldehydes in water was accomplished in an environment friendly approach for the synthesis of biscoumarins. Substituted biscoumarins on treatment with DMSO/acetic anhydride reagent under different conditions lead to various furocoumarins and the related compounds. Synthesis of furocoumarins was also attempted under microwave irradiation conditions starting from 3-bromo-4-hydroxycoumarin. The newly synthesized compounds were evaluated for their antiproliferative activity against human cancer cell line (HeLa) using MTT assay. **Results:** Synthesis of twenty-two structurally varied derivatives of 4-hydroxycoumarin has been accomplished. All the synthesized compounds were found to have better activity in comparison to the parent 4-hydroxycoumarin moiety. The compounds **(5a)**, **(5d)**, **(6a)**, **(6b)**, **(7a)**, **(8a)** and **(9a)** were found to exhibit good activity with an IC_{50} value below 100µM, the most promising being the compound **(6b)** with an IC_{50} value of 55µM. **Conclusion:** The various products synthesized from 4-hydroxycoumarin were found to have better activity in comparison to the starting compound.

Keywords: 4-hydroxycoumarin, biscoumarin, furocoumarin, anticancer

Introduction

The 4-hydroxycoumarin constitutes the structural nucleus for synthesis of a variety of products of pharmacological interest. Different derivatives of 4-hydroxycoumarin have been found to exhibit a variety of biological activities including anticoagulant (Abdelhafez et al., 2010; Au and Rettie, 2008), antibacterial (Brahmbhatt et al., 2013; Cespedes et al., 2006; El-Dean et al., 2013; Kidwai et al., 2014; Kumari et al., 2013; Musthafa et al., 2013), antifungal (Chohan et al., 2006; Rehman et al., 2005), antiviral (Zavrsnik et al., 2011), antioxidant (Jung and Park, 2009; Mladenovic et al., 2011; Vukovic et al., 2010a, 2010b), anti-inflammatory (Ahmad et al., 2009; Luchini et al., 2008) and anticancer (Bi et al., 2013; Jung et al., 2004; Kawaii et al., 2001; Stanchev et al., 2008) activity. 4-Hydroxycoumarin has been shown to exhibit antimetastatic effect *in vivo* and also effectively reduce tumor growth (Salinas-Jazmin et al., 2010). A series of 3-substituted-4-hydroxycoumarin derivatives has been synthesized and found potent against human cancer cell lines *in vitro* (Latif et al., 2016). Some carbonitrile and pyrazolyl derivatives of 4-hydroxycoumarin have been synthesized and evaluated for human breast adenocarcinoma and hepatocarcinoma cell lines and found to have moderate to weak cytotoxic activity (Abdel Hafiz et al., 2014).

In view of high degree of bioactivity shown by 4-hydroxycoumarin and the compounds derived from it, we report herein the synthesis of some of its derivatives and the comparative effect of structural changes on their anticancer activity.

Materials and Methods

The melting points were taken in open capillaries using a Perfit Melting Point apparatus and are uncorrected. The microwave synthesis reactor used was Anton Paar Monowave 300 and the reactions were carried out in a standard 10ml borosilicate glass vial (G10) or a large 30ml

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borosilicate glass vial (G30) sealed with a PTFE-coated silicone large 30ml borosilicate glass vial (G30) sealed with a PTFE-coated silicone septum closed with a snap cap. A teflon-coated stir bar was used to ensure proper homogenization of the reaction mixture during the reaction. Infrared spectra were recorded on a Perkin Elmer-Spectrum RX-IFTIR instrument. ^{1}H-NMR spectra were recorded on a Bruker Avance-II 400MHz NMR Spectrometer using $CDCl_3$ or DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. Mass Spectra were obtained on Waters Micromass Q-Tof Micro instrument. All the chemicals used were of AR grade. DMSO was distilled prior to the reaction and stored over 4Å molecular sieves.

The human cancer cell lines were procured from ATCC. The culture medium, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin and MTT reagent were purchased from Sigma Aldrich. The MTT assay was performed by recording the absorbance on a Microplate Reader (EPOCH, Bio-Tek Instruments, USA) at a wavelength of 590nm with background subtraction at 650nm.

Synthesis of 3-Bromo-4-hydroxycoumarin (1b)

4-Hydroxycoumarin **(1a)** (10 g) was dissolved in ethanol (100 mL) and kept in an ice-bath. Bromine (4 mL) was added dropwise to the ice-cold solution of 4-hydroxycoumarin. The reaction mixture was allowed to stand for half an hour. Then the mixture was added dropwise to ice and water (1000 mL) with vigorous stirring. The resulting slurry was kept overnight at room temperature. The crude 3-bromo-4-hydroxycoumarin **(1b)** was then filtered, washed with water and dried. It was recrystallized from ethyl acetate as white solid. Yield: 9.5g.

General procedure for synthesis of Biscoumarins (3a-I

A mixture of 4-hydroxycoumarin **(1a)** (4 mmol) and aldehydes **2a-i** (2 mmol) in 15 mL of 2:1 water-ethanol mixture was taken in a round-bottom flask and refluxed on a water bath. Progress of the reaction was monitored by thin layer chromatography. After completion of reaction, the mixture was cooled to room temperature. The products (**3a-**

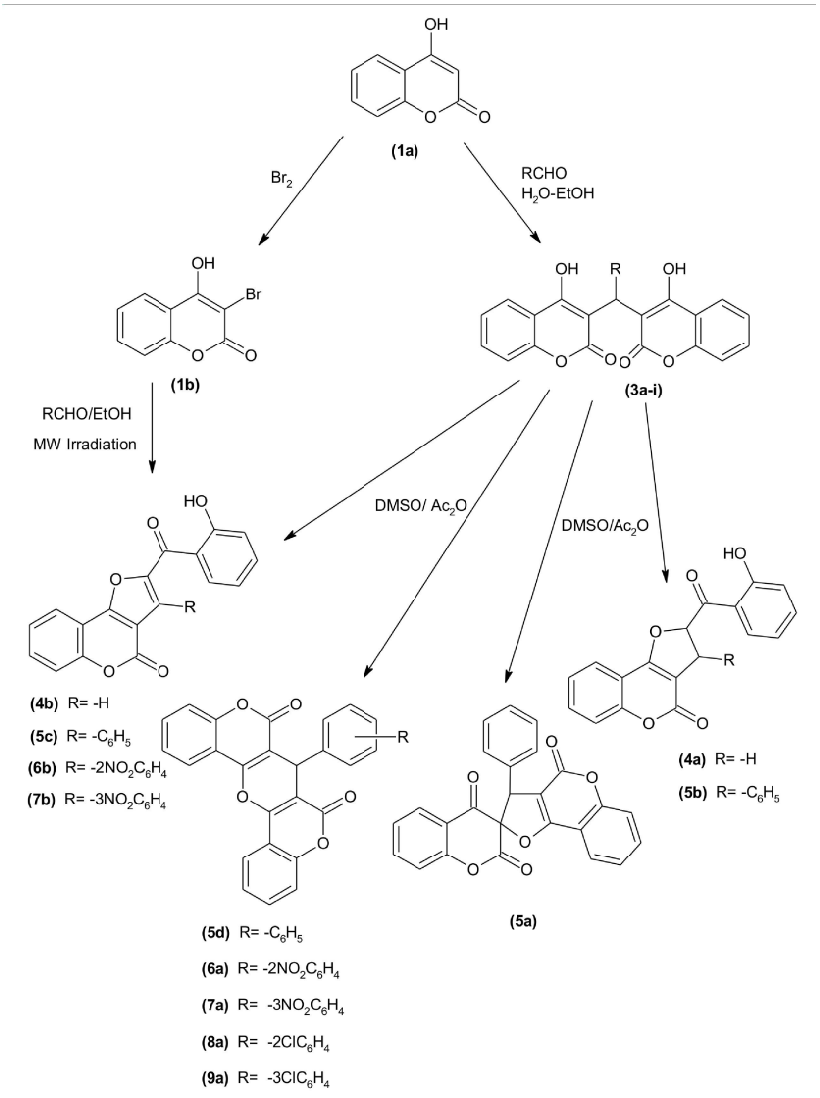

Figure 1. Various transformations of 4-hydroxycoumarin **(1a)**

i) were filtered, washed with ethanol, dried and used as such for further reactions.

General reaction of Biscoumarins (3a-h) with DMSO/Acetic anhydride at Room Temperature

A mixture of biscoumarin **(3a-h)** (2 mmol), DMSO (10 mL) and acetic anhydride (5 mL) was taken in a stoppered round-bottom flask and kept at room temperature for 3 days. The mixture was occasionally stirred and after the completion of reaction, water was added to the reaction mixture. The products (**4a-9a)** that precipitated out were filtered, washed with water, dried and then crystallized from an appropriate solvent.

Reaction of Dicoumarol (3a) with DMSO/Acetic anhydride at water bath temperature

A mixture of dicoumarol (**3a)** (2 mmol), DMSO (10 mL) and acetic anhydride (5 mL) was taken in a round-bottom flask and maintained on a boiling water bath. Progress of the reaction was monitored by thin layer chromatography. After completion of the reaction, the mixture was cooled to room temperature and diluted with water. The oily mass that separated out was extracted with ether. The product (**4a)** was filtered, washed with water, dried and crystallized from chloroform/petroleum ether as a white, feathery solid (m.p. 208-210°C). Yield: 0.461g.

Reaction of Biscoumarin (3c) with DMSO/Acetic anhydride at water bath temperature

A mixture of biscoumarin **(3c)** (2 mmol), DMSO (10 mL) and acetic anhydride (5 mL) was taken in a round-bottom flask and maintained on a boiling water bath for 6 hours. After completion of the reaction, the mixture was cooled to room temperature and diluted with water. The product (**5a)** that precipitated out was filtered, washed with water, dried and crystallized from benzene as white solid (m.p. 262-264°C). Yield: 0.583g.

Reaction of Biscoumarin (3c) with DMSO/Acetic anhydride at water bath temperature

A mixture of biscoumarin (**3c)** (2 mmol), DMSO (10 mL) and acetic anhydride (5 mL) was taken in a round-bottom flask and maintained on a boiling water bath for 24 hours. The reaction progress was monitored by thin layer chromatography. After completion of reaction, the mixture was cooled to room temperature and diluted with water. The products that precipitated out were filtered, washed with water, dried and separated by column chromatography using ethyl acetate/petroleum ether as eluent. Product (**5b)** was obtained as white prisms (m.p. 206-210°C). Yield: 0.302g and product (**5c)** as a yellow crystalline solid (m.p. 202-204°C). Yield: 0.400g.

General reaction of Biscoumarins (3d-h) with DMSO/Acetic anhydride at water bath temperature

A mixture of biscoumarin (**3d-h)** (2 mmol), DMSO (10 mL) and acetic anhydride (5 mL) was taken in a round-bottom flask and maintained on a boiling water bath for 24 hours. Progress of the reaction was monitored by thin layer chromatography. After completion of reaction, the mixture was cooled to room temperature and the insoluble products (**6a-9a)** were filtered, washed with water, dried and crystallized from benzene/chloroform. The filtrate was further diluted with water for the biscoumarins (**3d)** and (**3e)** and the products that precipitated out were filtered, washed with water and dried. The crude products (**6b-7b)** were purified by column chromatography using ethyl acetate/petroleum ether.

Reaction of Dicoumarol (3a) with DMSO/Acetic anhydride under microwave irradiation conditions

A mixture of dicoumarol (**3a)** (2 mmol), DMSO (6 mL) and acetic anhydride (3 mL) was taken in a borosilicate glass vial (G30) sealed with a PTFE-coated silicone septum closed with a snap cap. A teflon-coated stir bar was used to ensure proper homogenization of the reaction mixture during the reaction. The reaction mixture was irradiated at 150°C with heat as fast as possible option and a stirrer speed of 600 rpm for a hold time of 3 minutes. The cool down temperature was set at 55°C. After the completion of reaction, the mixture was diluted with water. The oily mass that precipitated out was extracted with ether. The product (**4a)** was filtered, washed with water and dried. It was further crystallized from chloroform/petroleum ether as a white feathery solid (m.p. 208-210°C). Yield: 0.549g.

Reaction of Biscoumarin (3c) with DMSO/Acetic anhydride under microwave irradiation conditions

A mixture of biscoumarin (**3c)** (2 mmol), DMSO (6 mL) and acetic anhydride (3 mL) was taken was taken in a borosilicate glass vial (G30) sealed with a PTFE-coated silicone septum closed with a snap cap. A teflon-coated stir bar was used to ensure proper homogenization of the reaction mixture during the reaction. The reaction mixture was irradiated at 150 °C with heat as fast as possible option and a stirrer speed of 600 rpm for a hold time of 3 minutes. The cool down temperature was set at 55°C. After the completion of reaction, the product (**5d)** which separated out was filtered, washed with water, dried and crystallized from benzene/chloroform as a white solid (m.p. >300°C). Yield: 0.206g.

Dilution of the filtrate with water resulted in precipitation of the product **(5c)** which was filtered, washed with water and dried. The crude product was purified by column chromatography using ethyl acetate/petroleum ether and obtained as yellow crystalline solid (m.p. 202-204°C). Yield: 0.482g.

Reaction of 3-Bromo-4-hydroxycoumarin (1b) with Formaldehyde (2a) under microwave irradiation conditions

A mixture of 3-bromo-4-hydroxycoumarin **(1b)** (2 mmol) and formaldehyde **(2a)** (1 mmol) in 3 mL ethanol was taken in a in a borosilicate glass vial (G10) sealed with a PTFE-coated silicone septum closed with a snap cap. A teflon-coated stir bar was used to ensure proper homogenization of the reaction mixture during the reaction. The reaction mixture was irradiated at 150°C with heat as fast as possible option and a stirrer speed of 600 rpm for a hold time of 2 minutes. The cool down temperature was set at 55 °C. After completion of reaction, the product that separated out was filtered, washed with ethanol and dried. The product (**4b)** was crystallized from benzene/petroleum ether as yellow needles (m.p. 182-184°C). Yield: 0.254g.

Reaction of 3-Bromo-4-hydroxycoumarin (1b) with Benzaldehyde (2c) under microwave irradiation conditions

A mixture of 3-bromo-4-hydroxycoumarin **(1b)** (2 mmol) and benzaldehyde **(2c)** (1 mmol) in 3 mL ethanol was taken in a borosilicate glass vial (G10) sealed with a PTFE-coated silicone septum closed with a snap cap. A teflon-coated stir bar was used to ensure proper homogenization of the reaction mixture during the reaction. The reaction mixture was irradiated at 150°C with heat as fast as possible option and a stirrer speed of 600 rpm for a hold time of 2 minutes. The cool down temperature was set at 55°C. After completion of reaction, the mixture was cooled to room temperature and diluted with water. The product (**5c)** that precipitated out was filtered under suction, washed with ethanol and dried. The crude product was purified by column chromatography using ethyl acetate/petroleum ether and obtained as yellow crystalline solid (m.p. 202-204°C). Yield: 0.289g.

Characterization of synthesized compounds

3-Bromo-4-hydroxy-2H-chromen-2-one (1b): White solid, m.p. 192-194°C; IR spectrum (KBr), ν (cm^{-1}): 3175 (O-H stretching), 1698, 1606 (C=O stretching), 1551, 1495, 1451 (C=C stretching of aromatic ring), 746 (C-H out-of-plane bending vibrations), 589 (C-Br stretching); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.72-7.98 (m, 4H, Ar-H); MS-ESI: calculated for [$C_9H_5BrO_3$-H]: 240.0, found: 239.9

3,3′-Methylenebis(4-hydroxy-2H-chromen-2-one) (3a): White crystalline solid; m.p. 278-280°C; IR spectrum (KBr), ν (cm^{-1}): 3062 (O-H stretching), 2904, 2800 (C-H stretching), 1639, 1599 (C=O stretching of lactone ring of coumarin), 1501, 1453 (C=C stretching of aromatic ring), 769, 750 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 3.82 (s, 2H, CH_2), 7.33-7.95 (m, 8H, Ar-H), 8.20 (s, 2H, OH); MS-ESI: calculated for [$C_{19}H_{12}O_6$-2H]: 334.2, found: 334.8

3,3′-Ethylidenebis(4-hydroxy-2H-chromen-2-one) (3b): White crystalline solid; m.p. 170-172°C; IR spectrum (KBr), ν (cm^{-1}): 3077, 3018 (O-H stretching), 2882 (C-H stretching), 1631 (C=O stretching of lactone ring of coumarin), 1568, 1496, 1450 (C=C stretching of aromatic ring), 803,761 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR ($CDCl_3$, 400MHz), δ (ppm): 1.85-1.87 (d, 3H, CH_3), 4.69-4.74 (m, 1H, CH), 7.26-8.00(m, 8H, Ar-H), 11.24 (s, 1H, OH), 12.05 (s, 1H, OH); MS-ESI: calculated for [$C_{20}H_{14}O_6$-2H]: 348.3, found: 348.9

3,3′-Benzylidenebis(4-hydroxy-2H-chromen-2-one) (3c): White crystalline solid; m.p. 226-228°C; IR spectrum (KBr), ν (cm^{-1}): 3064, 3023 (O-H stretching), 1654, 1618 (C=O stretching of lactone ring of coumarin), 1566, 1496, 1450 (C=C stretching of aromatic ring), 757 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR spectrum ($CDCl_3$, 400MHz), δ (ppm): 6.09 (s, 1H, CH), 7.20-8.07 (m, 13H, Ar-H), 11.30 (s, 1H, OH), 11.53(s, 1H, OH); MS-ESI: calculated for [$C_{25}H_{16}O_6$-2H]: 410.3, found: 410.9

3,3′-(2-Nitrobenzylidene)-bis(4-hydroxy-2H-chromen-2-one) (3d): Light yellow crystalline solid; m.p. 196-198°C; IR spectrum (KBr), ν (cm^{-1}): 3078, 3022 (O-H stretching), 1655, 1616 (C=O stretching of lactone ring of coumarin), 1565, 1495, 1454 (C=C stretching of aromatic ring), 1525, 1352 (NO_2 stretching), 763 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.71 (s, 1H, CH), 7.17-7.95 (m, 12H, Ar-H), 8.18 (s, 2H, OH); MS-ESI: calculated for [$C_{25}H_{15}NO_8$-2H]: 455.3, found: 455.9

3,3′-(3-Nitrobenzylidene)-bis(4-hydroxy-2H-chromen-2-one) (3e): Light yellow crystalline solid; m.p. 230-232°C; IR spectrum (KBr), ν (cm^{-1}): 3077, 3022 (O-H stretching), 1663, 1610 (C=O stretching of lactone ring of coumarin), 1566, 1496, 1449 (C=C stretching), 1530, 1347 (NO_2stretching), 764 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.58 (s, 1H, CH), 7.34-8.08 (m, 12H, Ar-H), 8.86 (s, 2H, OH); MS-ESI: calculated for [$C_{25}H_{15}NO_8$-2H]: 455.3, found: 455.9

3,3′-(4-Hydroxybenzylidene)-bis(4-hydroxy-2H-chromen-2-one) (3f): White crystalline solid; m.p. 208-210°C; IR spectrum (KBr), ν (cm^{-1}): 3356, 3076, 3020 (O-H stretching), 1648, 1616 (C=O stretching of lactone ring of coumarin), 1565, 1512, 1441 (C=C stretching), 762 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR spectrum ($CDCl_3$, 400MHz), δ (ppm): 6.01 (s, 1H, CH), 6.74-8.03 (m, 13H, Ar-H), 11.45 (s, 2H, OH); MS-ESI: calculated for [$C_{25}H_{16}O_7$-2H]: 426.3, found: 426.9

3,3′-(2-Chlorobenzylidene)-bis(4-hydroxy-2H-chromen-2-one) (3g): White crystalline solid; m.p. 196-198°C; IR spectrum (KBr), ν (cm^{-1}): 3072, 2981 (O-H stretching), 1646, 1617 (C=O stretching of lactone ring of coumarin), 1564, 1496, 1451 (C=C stretching), 761 (C-H out-of-plane bending vibrations of benzene ring), 641 (C-Cl stretching); ^{1}H NMR spectrum ($CDCl_3$, 400MHz), δ (ppm): 6.14 (s, 1H, CH), 7.21-8.03 (m, 12H, Ar-H), 10.93 (s, 1H, OH), 11.63 (s, 1H, OH); ESI-MS: calculated for [$C_{25}H_{15}ClO_6$-2H]: 444.8, found: 444.9

3,3′-(3-Chlorobenzylidene)-bis(4-hydroxy-2H-chromen-2-one) (3h): White crystalline solid; m.p. 222-224°C; IR spectrum (KBr), ν (cm^{-1}): 3074, 3019 (O-H stretching), 1658, 1617 (C=O stretching of lactone ring of coumarin), 1568, 1496, 1452 (C=C stretching), 761 (C-H out-of-plane bending vibrations of benzene ring), 651 (C-Cl stretching); ^{1}H NMR spectrum ($CDCl_3$, 400MHz), δ (ppm): 6.04 (s, 1H, CH), 7.09-8.07 (m, 12H, Ar-H), 11.30 (s, 1H, OH), 11.57 (s, 1H, OH); MS-ESI: calculated for [$C_{25}H_{15}ClO_6$-2H]: 444.8, found: 444.9

3-[6-oxo(1)benzopyran(4,3-b)-(1)benzopyran-7-yl]-4-hydroxy-2H-chromen-2-one (3i):

White crystalline solid; m.p. 240-242°C; IR spectrum (KBr), ν (cm^{-1}): 3003 (O-H stretching), 1696, 1617 (C=O stretching of lactone ring of coumarin), 1665, 1641 (C=C stretching of vinyl ether), 1569, 1488, 1454 (C=C stretching of aromatic ring), 1220, 1070 (C-O-C stretching of vinyl ether), 752 (C-H out-of-plane bending vibrations of benzene ring); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 5.78(s, 1H, CH), 7.08-8.08 (m, 12H, Ar-H), 11.99 (s, 1H, OH); MS-ESI: calculated for [$C_{25}H_{14}O_6$-H]: 409.3, found: 408.8

2,3-Dihydro-2-(2-hydroxybenzoyl)-4H-furo[3,2-c][1]benzopyran-4-one (4a): IR spectrum (KBr), ν (cm^{-1}): 3180 (O-H stretching), 1712 (C=O stretching), 1648, 1610 (C=O stretching of lactone ring of coumarin), 1571, 1501, 1488 (C=C stretching of aromatic ring), 1203, 1188 (C-O-C stretching of furan ring), 754, 730 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 3.08-3.68 (m, 2H, -CH_2), 6.59-6.64 (q, 1H, O=C-CH-O), 6.95-7.86 (m, 8H, Ar-H), 11.17 (s, 1H, OH); MS-ESI: calculated for [$C_{18}H_{12}O_5$-H]: 307.2, found: 307.0

2-(2-Hydroxybenzoyl)-4H-furo[3,2-c][1]benzopyran-4-one (4b): IR spectrum (KBr), ν (cm^{-1}): 3130 (O-H stretching), 1754 (C=O stretching), 1626, 1598 (C=O stretching of lactone ring of coumarin), 1560, 1537, 1485 (C=C stretching of aromatic ring), 1162, 1242 (C-O-C stretching of furan ring), 753 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 7.01-8.16 (m, 9H, Ar-H), 11.70 (s, 1H, OH); MS-ESI: calculated for [$C_{18}H_{10}O_5$-H]: 305.2, found: 305.0

Spiran (5a): IR spectrum (KBr), ν (cm^{-1}): 1792 (C=O stretching of saturated lactone), 1739, 1712 (C=O stretching of lactone ring of coumarin), 1661 (C=O stretching of benzoyl ketone), 1607, 1584, 1571 (C=C stretching of aromatic ring), 1047, 1098 (C-O-C stretching of furan ring), 764, 752 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 4.92 (s, 1H, Ar-CH-), 6.87-8.20 (m, 13H, Ar-H); MS-ESI: calculated for [$C_{25}H_{14}O_6$-H+H_2O-CO_2]: 383.3, found: 383.0

2,3-Dihydro-2-(2-hydroxybenzoyl)-3-phenyl-4H-furo[3,2-c][1]benzopyran-4-one (5b):

IR spectrum (Kbr), ν (cm^{-1}): 3434 (O-H stretching), 1720 (C=O stretching), 1656, 1619 (C=O stretching of lactone ring of coumarin), 1167, 1118 (C-O-C stretching of furan ring), 670,541 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 4.90 (d, 1H, Ar-CH-), 6.12 (d, 1H, O=C-CH-O-), 6.91-8.23 (m, 13H, Ar-H), 11.21 (s, 1H, OH); MS-ESI: calculated for [$C_{24}H_{16}O_5$-H-H_2]: 381.3, found: 381.3

2-(2-Hydroxybenzoyl)-3-phenyl-4H-furo[3,2-c][1]benzopyran-4-one (5c): IR spectrum (KBr), ν (cm^{-1}): 3406 (O-H stretching), 1730 (C=O stretching), 1630, 1625 (C=O stretching of lactone ring of coumarin), 1175, 1216 (C-O-C stretching of furan ring); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.76-8.07 (m, 13H, Ar-H), 11.72 (s, 1H, OH); MS-ESI: calculated for [$C_{24}H_{14}O_5$-H]: 381.3, found: 381.0

7-Phenyl-7H-bis-[1]benzopyrano[4,3-b:3′,4′-c]pyran-6,8-dione (5d): IR spectrum (KBr), ν (cm^{-1}): 1657, 1619 (C=O stretching of lactone ring of coumarin), 1166, 1119 (C-O-C stretching of pyran ring), 670 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 5.24 (s, 1H, Ar-CH-), 7.13-9.26 (m, 13H, Ar-H); MS-ESI: calculated for [$C_{25}H_{14}O_5$-H+H_2O]: 411.3, found: 411.0

7-(2-Nitrophenyl)-7H-bis-[1]benzopyrano[4,3-b:3′,4′-c]pyran-6,8-dione (6a): White solid; m.p. 294-296°C; IR spectrum (KBr), ν (cm^{-1}): 1671, 1619 (C=O stretching of lactone ring of coumarin), 1529, 1359 (NO_2stretching), 1149, 1011 (C-O-C stretching of pyran ring), 669 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.21 (s, 1H, Ar-CH-), 7.46-8.72 (m, 12H, Ar-H); MS-ESI: calculated for [$C_{25}H_{13}NO_7$-H+H_2O]: 456.3, found: 456.0

2-(2-Hydroxybenzoyl)-3-(2-nitrophenyl)-4H-furo[3,2-c][1]benzopyran-4-one (6b): Yellow crystalline solid; m.p. 208-210°C; IR spectrum (KBr), ν (cm^{-1}): 3398 (O-H stretching), 1764 (C=O stretching), 1668, 1625 (C=O stretching of lactone ring of coumarin), 1535, 1359 (NO_2 stretching), 1210, 1160 (C-O-C stretching of furan ring), 732 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 7.33-8.85 (m, 12H, Ar-H),

11.36 (s, 1H, OH); MS-ESI: calculated for [$C_{24}H_{13}NO_7$-H]: 426.3, found: 426.0

7-(3-Nitrophenyl)-7H-bis-[1]benzopyrano[4,3-b:3′,4′-c]pyran-6,8-dione (7a): White solid; m.p. 278-280°C; IR spectrum (KBr), ν (cm^{-1}): 1657, 1620 (C=O stretching of lactone ring of coumarin), 1530, 1351 (NO_2 stretching), 1144, 1005 (C-O-C stretching of pyran ring), 667 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.39 (s, 1H, Ar-CH-), 6.94-8.42 (m, 12H, Ar-H); MS-ESI: calculated for [$C_{25}H_{13}NO_7$-H+H_2O]: 456.3, found: 456.0

2-(2-Hydroxybenzoyl)-3-(3-nitrophenyl)-4H-furo[3,2-c][1]benzopyran-4-one (7b): Yellow crystalline solid; m.p. 194-196°C; IR spectrum (KBr), ν (cm^{-1}): 3420 (O-H stretching), 1761 (C=O stretching), 1655, 1629 (C=O stretching of lactone ring of coumarin), 1537, 1349 (NO_2 stretching), 1221, 1158 (C-O-C stretching of furan ring), 746 (C-H out-of-plane bending vibrations); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 6.93-8.52 (m, 12H, Ar-H), 11.51 (s, 1H, OH); MS-ESI: calculated for [$C_{24}H_{13}NO_7$-H]: 426.3, found: 426.0

7-(2-Chlorophenyl)-7H-bis-[1]benzopyrano[4,3-b:3′,4′-c]pyran-6,8-dione (8a): White solid; m.p. 268-270°C; IR spectrum (KBr), ν (cm^{-1}): 1653, 1620 (C=O stretching of lactone ring of coumarin), 1147, 1021 (C-O-C stretching of pyran ring), 645 (C-Cl stretching); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 5.28 (s, 1H, Ar-CH-), 6.96-8.12 (m, 12H, Ar-H); MS-ESI: calculated for [$C_{25}H_{13}ClO_5$-H+H_2O]: 445.8, found: 446.0

7-(3-Chlorophenyl)-7H-bis-[1]benzopyrano[4,3-b:3′,4′-c]pyran-6,8-dione (9a): White solid; m.p. 236-238°C; IR spectrum (KBr), ν (cm^{-1}): 1667, 1621 (C=O stretching of lactone ring of coumarin), 1151, 1029 (C-O-C stretching of pyran ring), 649 (C-Cl stretching); ^{1}H NMR spectrum (DMSO-d_6, 400MHz), δ (ppm): 5.27 (s, 1H, Ar-CH-), 6.98-8.01 (m, 12H, Ar-H); MS-ESI: calculated for [$C_{25}H_{13}ClO_5$-H+H_2O]: 445.8, found: 446.0

Cell Culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum, 50µg/ml penicillin and 100µg/ml streptomycin in a humidified atmosphere of 37°C. Cells were routinely passaged twice a week. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.5% in PBS containing 0.02% EDTA).

MTT Assay

HeLa cells were plated in 12-well plates in cell culture medium (DMEM) and grown overnight at 37°C in CO_2 incubator. The cells were treated with various concentrations of the compounds (0, 20, 30, 60, 80µg/mL) and incubated for 24 hours. The cytotoxicity was measured by MTT assay. 100µl of MTT (5 mg/mL) was added to each well and plates were further incubated at 37°C for 3 hours. The MTT solution was gently pipetted out (aspirated) and 150 µl of DMSO was added to cells, followed by incubation at room temperature for 30 minutes. The absorbance was measured at a wavelength of 590nm with background subtraction at 650nm using a microplate reader.

The experiments were repeated thrice, and the results were presented as the percentage of cell viability, calculated using the formula given below:

% Cell Viability = (Absorbance of treated cells/ Absorbance of untreated cells) × 100

A dose-response curve was plotted for each test compound and the half maximal inhibitory concentration (IC_{50}) values, corresponding to the concentration of compound that inhibits 50 % of the cell growth, were determined.

Results and discussion

Various derivatives of 4-hydroxycoumarin **(1a)** were synthesized. First, we attempted bromination of 4-

Table 1. Synthesis of biscoumarins **(3a-h)** under conventional reflux in H_2O-EtOH (2:1 ratio) as solvent[a]

-R	Aldehydes	Reflux Time/ hrs	Product	Yield[b]/ %
-H	Formaldehyde **(2a)**	4	**3a**	85.25
$-CH_3$	Acetaldehyde **(2b)**	24	**3b**	43.74
$-C_6H_5$	Benzaldehyde **(2c)**	12	**3c**	78.42
2-$NO_2C_6H_4$	*o*-Nitrobenzaldehyde **(2d)**	**7**	**3d**	74.62
3-$NO_2C_6H_4$	*m*-Nitrobenzaldehyde **(2e)**	9	**3e**	89.80
4-HOC_6H_4	*p*-Hydroxybenzaldehyde**(2f)**	24	**3f**	70.92
2-ClC_6H_4	*o*-Chlorobenzaldehyde **(2g)**	13	**3g**	88.68
3-ClC_6H_4	*m*-Chlorobenzaldehyde **(2h)**	2	**3h**	90.74
2-HOC_6H_4	*o*-Hydroxybenzaldehyde **(2i)**	3	**3i**	81.64

[a]Reaction conditions: 4 mmol 4-hydroxycoumarin, 2 mmol aldehyde, 15 mL solvent. [b]Isolated yield

hydroxycoumarin. Synthesis of 3-bromo-4-hydroxycoumarin **(1b)** was carried out by the method already reported in literature (Eisenhauer and Link, 1954)**.** We then carried out the synthesis of various biscoumarins starting from 4-hydroxycoumarin and different aldehydes in a simple procedure (Banday et al., 2017) using water as solvent (Table 1).

Finally, the reaction of substituted biscoumarins with DMSO/acetic anhydride reagent was carried out using conventional as well as microwave-assisted synthesis and the conditions were manipulated to get wide range of products. Dicoumarol reacts with DMSO and acetic anhydride at 80°C (Khan et al., 1983) to give 2,3-dihydro-2-(2-hydroxybenzoyl)-4*H*-furo[3,2-*c*][1]benzopyran-4-one and 2-(2-hydroxybenzoyl)-4*H*-furo [3,2-*c*][1]benzopyran-4-one. This reaction was manipulated for the synthesis of substituted furo[3,2-*c*]coumarins and related compounds by using various substituted biscoumarins instead of dicoumarol (Table 2).

The reaction of 3-bromo-4-hydroxycoumarin **(1b)** with aldehydes in ethanol (Rehman et al., 1990) was explored further and attempts were made to carry out the reaction under microwave irradiation conditions. The reaction was successful for the aldehydes, formaldehyde **(2a)** and benzaldehyde **(2c)** where the corresponding furocoumarins **(4b)** and **(5c)** were obtained (Table 3).

All the compounds were evaluated for their *in vitro* cytotoxicity against human cancer cell lines (HeLa) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The cells were allowed to proliferate in presence of different concentrations of the test materials for 24 hours. The results are presented as the percentage of cell viability (Table 4), calculated using the formula given below:

% Cell Viability = (Absorbance of treated cells/ Absorbance of untreated cells) × 100

A dose-response curve was plotted for each test compound and the half maximal inhibitory concentration (IC_{50}) values, corresponding to the concentration of compound that inhibits 50 % of the cell growth, were determined (Table 5).

The cytotoxicity of 4-hydroxycoumarin **(1a)** was compared with 3-bromo-4-hydroxycoumarin **(1b)**, various

Table 2. Various Products obtained under different conditions

S. No	Reactant	Reagent	Conditions	Time	Products [Yield[a] /g]
1.	**(3a)**	DMSO/Ac_2O	Room Temp.	3 Days	**(4a)** [0.384]
			Water Bath	12 hours	**(4a)** [0.461]
			MW Irradiation	3 minutes	**(4a)** [0.549]
2.	**(3c)**	DMSO/Ac_2O	Room Temp.	3 Days	**(5a)** [0.621]
			Water Bath	6 hours	**(5a)** [0.583]
			Water Bath	24 hours	**(5b)** [0.302] + **(5c)** [0.400]
			MW Irradiation	3 minutes	**(5d)** [0.206] + **(5c)** [0.482]
3.	**(3d)**	DMSO/Ac_2O	Room Temp.	3 Days	**(6a)** [0.674]
			Water Bath	24 hours	**(6a)** [0.356] and **(6b)** [0.402]
			MW Irradiation	3 minutes	**(6a)** [0.321] and **(6b)** [0.474]
4.	**(3e)**	DMSO/Ac_2O	Room Temp.	3 Days	**(7a)** [0.612]
			Water Bath	24 hours	**(7a)** [0.342] and **(7b)** [0.394]
			MW Irradiation	3 minutes	**(7a)** [0.284] and **(7b)** [0.456]
5.	**(3g)**	DMSO/Ac_2O	Room Temp.	3 Days	**(8a)** [0.638]
			Water Bath	24 hours	**(8a)** [0.605]
6.	**(3h)**	DMSO/Ac_2O	Room Temp.	3 Days	**(9a)** [0.665]
			Water Bath	24 hours	**(9a)** [0.623]

[a]Isolated yield

Table 3. The reaction of 3-bromo-4-hydroxycoumarin (1b) with aldehydes in ethanol[a]

Reactants		Conditions	Time	Yield[b]/g
3-Bromo-4-hydroxycoumarin **(1b)**	HCHO **(2a)**	MW Irradiation	2 minutes	**(4b)** [0.254]
	C_6H_5CHO **(2c)**			**(5c)** [0.289]

[a]Reaction conditions: 2 mmol 3-Bromo-4-hydroxycoumarin, 1mmol aldehyde, 3 mL ethanol. [b]Isolated yield

Table 4. % Cell viabilty of the various compounds at different concentrations

Compounds/ Concentration (µg/ml)	% Cell Viability			
	20	30	60	80
1a	93.82	92.92	68.03	51.38
1b	90.46	78.02	46.06	24.85
3a	90.23	87.27	58.28	39.05
3b	95.52	77.63	51.72	34.52
3c	92.94	84.33	53.50	32.94
3d	95.75	88.78	59.01	39.09
3e	98.23	91.17	55.32	31.76
3f	86.33	77.00	45.40	24.33
3g	87.33	87.00	48.01	22.00
3h	89.66	85.24	48.32	23.66
3i	99.12	90.35	56.35	33.62
4a	92.64	66.47	46.01	32.35
4b	71.33	59.33	31.69	13.33
5a	70.92	59.61	31.36	12.66
5b	97.33	69.00	36.93	15.66
5c	85.33	63.00	33.95	14.66
5d	60.33	58.00	30.77	12.66
6a	78.33	56.33	31.09	14.33
6b	54.66	41.33	27.88	19.00
7a	74.00	60.33	31.48	12.33
7b	95.00	74.33	37.73	13.33
8a	67.00	62.00	33.18	14.00
9a	64.00	60.46	31.78	12.66

Table 5. IC_{50} values of the various synthesized compounds

Compounds	IC_{50} Values/ µM
1a	504
1b	233
3a	203
3b	177
3c	153
3d	151
3e	141
3f	129
3g	130
3h	131
3i	159
4a	175
4b	130
5a	**97**
5b	124
5c	113
5d	**98**
6a	**85**
6b	**55**
7a	**92**
7b	116
8a	**99**
9a	**95**

biscoumarins and the furocoumarins and related compounds. It is apparent that bromination of 4-hydroxycoumarin leads to a decrease in IC_{50} value from 504µM to 233µM. The biscoumarins with different substitutions have different IC_{50} values with the simplest dicoumarol **(3a)** having an IC_{50} value of 203µM which decreases for various substituted biscoumarins and the compound **(3f)** having the least IC_{50} value of 129µM. The furocoumarins and related compounds have a further lower IC_{50} value and hence better cytotoxicity when compared with the corresponding biscoumarins. Thus, the cytotoxicity of 4-hydroxycoumarin is enhanced by various transformations.

Conclusion

In conclusion, synthesis of various structurally different derivatives of 4-hydroxycoumarin has been carried out so as to study the effect on anticancer activity. All the synthesized compounds were evaluated for their antiproliferative activity against human cancer cell line (HeLa) using MTT assay and were found to have enhanced activity in comparison to the 4-hydroxycoumarin **(1a)**. Among the biscoumarins, the compound **(3f)** was found to have the best activity with an IC_{50} value of 129µM. The various products synthesized from biscoumarins were found to have better activity in comparison to the starting compounds. Overall, the compounds **(5a)**, **(5d)**, **(6a)**, **(6b)**, **(7a)**, **(8a)** and **(9a)** were found to exhibit best activity with an IC_{50} value below 100µM. The most promising compound was found to be the compound **(6b)** with an IC_{50} value of 55µM.

Conflicts of interest

The authors declare no conflicts of interest.

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