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Design, synthesis, biological and *in silico* evaluation of coumarin-hydrazone derivatives as tubulin targeted antiproliferative agents

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ABSTRACT

Coumarin-based different series of hydrazone derivatives were synthesized and evaluated for anticancer activity against four different human cancer cell lines. The activity of the compounds were compared with doxorubicin as a standard drug and all the compounds exhibited good to moderate cytotoxicity with IC_{50} values ranging from 6.07 to 60.45 μM against all the examined cancer cell lines. Based on the screening results, it was concluded that the compounds **12a** and **18a** were the most promising medicinal entities. *In vitro* tubulin polymerisation inhibition assay was performed for the compounds **12a** and **18a** and these two compounds displayed good potency when compared with colchicine as the standard drug. The interaction of these compounds with tubulin protein was also studied with the help of molecular docking technique using Discovery studio software. Furthermore, the molecular and ADMET properties of the compounds were computed with Osiris property software and PreADMET server. The compounds exhibited exciting *in vitro* and *in silico* results. Hence we propose that the compounds **12a** and **18a** could be developed as tubulin targeted potential antiproliferative agents.

1. Introduction

The design and optimization of anticancer agents with the acceptable pharmacodynamics, toxicological and pharmacokinetic [1,2] are the fundamental focuses of medicine development. Using *in silico* methods for prediction of these characteristics in the initial evolution stages is extremely beneficial for the drug innovation process. Identification of the right target and a strong mechanism of action would effectively reduce the time span in the drug discovery process [3]. The effective molecules can be thoroughly examined in computational models even before molecular synthesis stage. Furthermore, pharmacokinetic studies can provide useful insights into the behaviour of absorption, distribution and pathway of drug metabolism with biological sites (e.g. cytochrome P450 isoforms) [4]. *Lipinski's rule* may be used as decision gate to facilitate the development of orally bioavailable drugs [5]. Molecular docking is one of the highly used computational techniques to have an idea of the suitable target(s) and active sites of the potential drug molecules.

Cancer is one of the life-threatening diseases which affects almost 60 human organs [6]. It is a highly heterogeneous and complex disease caused by some etiological agents, many of which are unknown. No single chemotherapeutic agent has been 100% successful in the treatment of cancer. Moreover, existing chemotherapeutic agents are progressively becoming less effective due to development of drug-resistance by the cancer cells [7], along with the side effects such as hepatotoxicity, myelotoxicity, neurotoxicity, pulmonary toxicity, urinary toxicity and cardiac toxicity upon their long-term use. Various research groups around the globe are working to produce efficient anticancer drugs with high effectiveness and low side effects [8].

Coumarins are a category of naturally occurring organic compounds (mainly found in plants) with several medicinal properties [9]. These compounds belong to the benzopyrones class and are one of the significant sources for the new anticancer agents [10]. Coumarins have been reported to have a wide variety of pharmacological activities such as antibacterial [11], antialzheimer [12], antidiabetic [13], antioxidant [13], anticoagulant [14], anti-inflammatory, analgesic [15] etc. Various research groups around the world have reported many anticancer agents with coumarin moeity. Nasr et al. in 2014 reported 6-brominated coumarin derivatives that exhibited potential anticancer activity against Hep-G2 and CCRF cell lines [16]. Further in 2017 Batran et al. reported 4-hydroxy coumarins and their anticancer behaviour against breast cancer (MCF-7) and cervical cancer (HeLa) [17]. Coumarin sulphonamides demonstrated their anticancer activity against HeLa cell lines [18]. From the previous reports we observed that toluenesulfonyl, benzoyl and isoniazid hydrazone derivatives showed significant anticancer activity [19–22].

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Tubulin protein is one of the important targets in the treatment of cancer due to its involvement in wide variety of cellular processes like mitosis, cell division, intracellular transport, cell shape and migration in eukaryotic cells [23]. Tubulin targeted drugs e.g. vinca alkaloids, colchicine derivatives and podophyllotoxins are having prominent use in the treatment of cancer [24,25].

The potential significance of coumarins as well as hydrazone derivatives encouraged us to design, synthesize new coumarin based hydrazone derivatives and to investigate their potential for *in vitro* antiproliferative activity and *in vitro* tubulin polymerisation inhibition assay. In this present work, we have also performed molecular docking studies, *in silico* molecular properties, ADMET evaluation studies for all synthesized compounds.

2. Materials and methods

All reactions were carried out in 100 mL and 250 mL RBFs (round bottom flask). All the starting materials were purchased from Sigma and Himedia and used without further purification. Dulbecco's modified Eagle medium (DMEM), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], trypsin-EDTA, antibiotic-antimycotic solution, phosphate buffered saline (Ca^{2+}, Mg^{2+} free; PBS), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Foetal bovine serum (FBS) was purchased from Gibco, USA. Cell culture 96 well plates and plastic wareswere obtained from Techno Plastic Products (TPP) (CH-8219, Trasadingen, Switzerland). All other chemicals were obtained locally and were of analytical grade.

The progress of the reactions was monitored by thin layer chromatography (TLC) with ethyl acetate-hexane (1:1) mixture as eluent. Melting points of the compounds were recorded on a Digital Auto Melting/Boiling point apparatus (LABARD LIM-252) and are uncorrected. FT-IR spectra were recorded on RX-I Perkin Elmer Instrument using KBr pellet. All NMR spectra were recorded on (400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR) Bruker FT-NMR spectrometer and chemical shifts were expressed in δ units relevant to Tetra methyl silane (TMS) signal as an internal source in DMSO-d_6. Signals were indicated as s (singlet), d (doublet), t (triplet) m (multiplet, when multiplicity is complex) for ^{1}H NMR. Coupling constants, *J* was recorded in Hz. Mass of the synthesized compounds were recorded by Liquid chromatography-Mass spectrometry (LC-MS; Perkin Elmer, Flexar SQ 300 ms).

2.1. Chemistry

2.1.1. Synthesis of compound **3**

A mixture of R-Ph-OH (10 mmol), Malonic acid (10 mmol), $ZnCl_2$ (30 mmol) and $POCl_3$ (30 mmol) was stirred at 70 °C for 24 hrs. Then it was allowed to cool and poured into ice-cold water. The precipitate was collected, washed several times with water, dissolved in 1 N NaOH, and the insoluble suspension was removed by filtration. The filtrate was acidified with concentrated HCl, and the precipitate was collected. The crude compound was crystallized from methanol solvent.

2.1.2. Synthesis of compound **4**

In 250 mL RB flask 3 g of compound **3** was taken, and to this 15 mL of acetic acid and 5 mL of $POCl_3$ were added; the mixture was refluxed for 3 hrs. Then the solution was poured into ice-cold water. The precipitate was collected and washed several times with distilled water. Then the product was purified by chromatography (1:1, ethyl acetate: hexane) to have white colour solid with 75% yield [26].

2.1.3. General procedure for the synthesis of compounds (**6a–c, 12a–c** and **18a–c**)

To the 1 mmol of compound **4** in a 100 mL RB flask, 1 mmol of compound ***5*** was added and dissolved in ethanol. To the reaction mixture added few drops of acetic acid, the contents of flask was allowed to stirr for 2 hrs, at 60 °C. After completion of the reaction, the mixture was poured into ice-cold water; the residue was collected by filtration, and then recrystallized from ethanol/DMF (4:1) solvent mixture to afford the final desired compounds [26].

2.1.3.1. N′-(1-(4-hydroxy,7-methyl-2-oxo-2H-chromen-3-yl)ethylidene) benzohydrazide ***(6a)***. Light yellow colour solid, yield (58%) M.P: 210–212 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3502, 3116, 1670, 1607; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 15.71 (s, 1H), 11.81 (s, 1H), 7.85 (d, *J* = 7.2 Hz, 2H),7.78 (d, *J* = 8 Hz, 2H), 7.61 (t, *J* = 7.2 Hz, 1H), 7.55 (t, *J* = 7.6 Hz, 2H),7.27 (d, *J* = 2 Hz, 1H) 7.10 (m, 2H), 2.51 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 179.54, 172.78, 153.43, 145.88, 140.50, 133.21, 131.52, 129.18, 128.39, 127.54, 125.93, 125.59, 116.49, 115.11, 95.48, 21.62, 18.15; ESI-MS [M + Na]: 359.79 Da.

2.1.3.2. N′-(1-(4-hydroxy,7-methyl-2-oxo-2H-chromen-3-yl)ethylidene) methylbenzene sulfonohydrazide ***(6b)***. White colour solid, yield (55%), M.P: 220–222 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3419, 3206, 1732, 1625, 1569;^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 14.85 (s, 1H), 9.59 (s, 1H), 7.82 (d, *J* = 8.4 Hz, 2H), 7.64 (d, *J* = 8 Hz, 2H), 7.38 (d, *J* = 8.4 Hz, 1H), 7.27 (d, *J* = 2 Hz), 7.20 (m, 1H), 2.50 (s, 3H), 2.44 (s, 3H) , 2.30 (s, 3H) ; ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 169.22, 160.02, 153.90, 153.35, 153.28, 143.84, 135.86, 129.83, 128.19, 126.80, 118.83, 117.89, 114.12, 110.51, 21.42, 21.27, 18.56; ESI-MS [M + Na]: 409.56 Da.

2.1.3.3. N′-(1-(4-methyl,7-hydroxy-2-oxo-2H-chromen-3-yl)ethylidene) isonicotinohydrazide ***(6c)***. Orange colour solid, yield (62%), M.P: 236–238 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3485, 3065, 2678, 1677, 1559; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 14.85 (s, 1H), 10.81 (s, 1H), 7.95 (d, *J* = 7.2 Hz, 2H), 7.88(d, *J* = 8 Hz, 2H), 7.66 (t, *J* = 7.2 Hz, 1H), 7.57 (t, *J* = 7.6 Hz, 2H), 7.28 (d, 1H), 2.38 (s, 3H) , 2.14 (s, 3H) ;^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 177.35, 152.21, 145.82, 143.98, 135.20, 132.52, 129.35, 128.93, 127.26,125.34, 123.62, 116.98, 115.21, 98.31, 21.65, 18.95; ESI-MS [M + Na]: 360.7.

2.1.3.4. N′-(1-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)ethylidene) benzohydrazide ***(12a)***. White colour solid, yield (76%), M.P: 230–232 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3454, 3256, 3084, 1692, 1611; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 15.69 (s, 1H), 11.85 (s, 1H), 8.02 (d, *J* = 12 Hz, 1H), 7.95 (t, *J* = 7.2 Hz, 2H), 7.66 (d, *J* = 7.6 Hz, 1H), 7.58 (t, *J* = 8 Hz, 2H), 7.14 (m, 2H), 2.75 (s, 3H), 2.31 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 179.09, 172.59, 169.11, 165.28, 155.15, 154.13, 133.13, 131.54, 129.12, 128.35, 127.40, 118.48, 117.88, 110.23, 95.37, 21.31; ESI-MS [M + Na]: 361.58 Da.

2.1.3.5 N′-(1-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)ethylidene)4-methylbenzenesulphonyl hydrazide ***(12b):*** White colour solid, yield (70%), M.P: 248–250 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3436, 3076, 2826, 1769, 1616, 1563; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 15.64 (s, 1H), 11.83 (s, 1H), 8.01 (d, *J* = 8.4 Hz, 1H),7.93 (d, *J* = 7.2 Hz, 2H), 7.64 (d, *J* = 7.6 Hz,1H), 7.56 (t, *J* = 7.6 Hz, 2H), 7.12 (m, 2H), 2.73 (s, 3H), 2.29 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 179.10, 172.68, 169.10, 155.18, 154.15, 133.12, 131.57, 129.12, 128.34, 127.42, 118.50, 117.90, 110.24, 95.39, 21.31, 18.09; ESI-MS [M + Na]: 411.58 Da.

2.1.3.6 N′-(1-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)ethylidene)isonicotinohydrazide ***(12c):*** Orange colour solid, yield (85%), M.P: 242–244 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3195, 3067, 2287, 1663, 1596; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 15.75 (s, 1H), 10.85 (s, 1H), 8.83 (t, *J* = 1.2 Hz, 2H), 8.02 (d, *J* = 8.4 Hz 1H), 7.95 (s, 2H), 7.13 (m, 2H), 2.74 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 176.32, 167.25, 155.21, 154.35, 145.28,136.73, 132.55, 131.80, 127.28, 126.95, 119.34, 115.41, 107.29, 96.42, 26.01.

ESI-MS [M + Na]: 362.68 Da.

2.1.3.5. N′-(1-(4-hydroxy-2-oxo-2H-Chromen-3-yl)ethylidene) benzohydrazide ***(18a)***. White colour solid, yield (82%), M.P: 222–224 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3408, 3217, 1706, 1650; ^{1}H NMR (400 MHz,

DMSO-d_6, δ ppm) 15.72 (s, 1H), 11.83 (s, 1H), 7.99 (d, J = 1.6 Hz, 1H), 7.95 (m, 2H), 7.65 (m, 2H), 7.56 (t, J = 8 Hz, 2H), 7.31 (m, 2H), 2.74 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 179.71, 172.68, 165.30, 153.54, 134.81, 133.14, 131.56, 129.14, 128.35, 126.12, 124.32, 120.05, 116.78, 95.72, 18.10; ESI-MS [M + Na]: 345.53 Da.

2.1.3.8 N′-(1-(4-hydroxy-2-oxo-2H-chromen-3-yl)ethylidene)4-methylbenzenesulphonyl hydrazide ***(18b):*** White colour solid, yield (80%), M.P: 212–214 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3443, 3065, 2826, 1677, 1566; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 14.82 (s, 1H), 7.84 (m, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.63 (m, 1H), 7.48 (d, J = 8 Hz, 2H), 7.26 (m, 2H), 2.60 (s, 3H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 179.28, 177.55, 166.10, 153.44, 145.47, 135.22, 134.01, 130.66, 128.13, 126.10, 124.44, 119.22, 116.79, 97.30, 21.54, 18.02; ESI-MS [M + Na]: 395.66 Da.

2.1.3.6. N′-(1-(4-hydroxy-2-oxo-2H-chromen-3-yl)ethylidene) isonicotinohydrazide ***(18c)***. Orange colour solid, yield (85%), M.P: 244–246 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3417, 3084, 2659, 1677, 1559; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 15.74 (s, 1H), 8.82 (d, J = 5.6 Hz, 2H),7.95 (m, 3H), 7.54 (t, J = 8.4 Hz, 1H), 7.30 (m, 2H), 2.75 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 178.32, 173.16, 169.23, 155.46, 154.29, 133.72, 131.53, 129.96, 128.74, 127.45, 118.51, 117.56, 110.68, 96.35,18.29; ESI-MS [M + Na]: 346.81 Da.

2.1.4. Synthesis of (7-hydoxy-4-methyl)-2H-Chromen-2-one ***(21)***

A mixture of *m*-cresol (10 mmol), ethyl acetoacetate (10 mmol), and added H_2SO_4 (5 mmol) reacted at 10 °C for 2.5 hrs. The precipitate was collected, washed several times with water, dissolved in 1 N NaOH, and the insoluble suspension was removed by filtration. The filtrate was acidified with concentrated HCl, and the precipitate was collected. Crude compound was recrystallized using methanol (yield 85%). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 11.62 (s, 1H, OH), 8.02 (d, J = 8.2 Hz, 1H), 6.88 (m, 1H), 6.76 (d, J = 8 Hz, 1H), 6.42 (s, 1H), 3.38 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 161.96, 161.05, 156.29, 151.26, 125.96, 115.31, 110.55, 108.23, 102.71, 18.98.

2.1.5. Synthesis of (3-acetyl-7-hydroxy-4-methyl)-2H-Chromen-2-one ***(22)***

To the 5 g of compound ***21,*** 25 mL of acetic acid and 8 mL of $POCl_3$ was added in 250 mL RBF. Refluxed the mixture for 3 hrs and poured the hot reaction mixture into ice-cold water then collected the precipitate and washed with water for 3 times. Light yellow colour solid, yield (72%): ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 10.43 (s, 1H,), 7.96 (d, J = 7.2 Hz, 1H),7.83 (d, J = 8 Hz, 2H), 5.85 (s, 1H), 2.70 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 179.72, 167.53, 153.30, 138.12, 125.30, 123.26, 116.67, 116.42, 113.53, 96.25, 23.05, 21.75.

2.1.6. General method for the synthesis of compounds ***(24a–c)***

To the solution of **22** (1 mmol) in ethanol (20 mL) in 100 mL RB (round bottom) flask, added 1 mmol of compound ***5*** and few drops of acetic acid, stirred the mixture for 2 hrs at 60 °C. After completion of the reaction, the mixture was poured into ice-cold water then filtered and the precipitate was collected by vacuum filtration. Further the precipitate was recrystallized from ethanol/DMF (4:1) solvent mixture to afford final desired compounds.

2.1.6.1 N′-(1-(7-hydroxy-4-methyl-2-oxo-2H-chromen-3-yl)ethylidene) benzohydrazide ***(24a):*** White colour solid, yield (65%), M.P: 218–220 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3452, 3047, 1668, 1556; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 15.71 (s, 1H), 11.79 (s, 1H), 7.95 (d, J = 7.2 Hz, 2H), 7.88 (d, J = 8 Hz, 1H), 7.66 (t, J = 7.2 Hz, 1H), 7.57 (t, J = 7.6 Hz, 2H), 7.13 (m, 2H), 2.49 (s, 3H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm) 179.74, 172.69, 153.55, 145.82, 140.60, 133.11, 131.58, 129.14, 128.29, 127.59, 125.96, 125.49, 116.65, 115.08, 95.44, 21.64, 18.11; ESI-MS [M + Na]: 359.68 Da.

2.1.6.2 N′-(1-(7-hydroxy-4-methyl-2-oxo-2H-chromen-3-yl)ethylidene) 4-methylbenzenesulfonohydrazide ***(24b):*** White colour solid, yield (72%), M.P: 224–226 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3417, 3074, 1668, 1663, 1559; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 14.93 (s, 1H), 10.81 (s, 1H), 7.31 (t, J = 8.4 Hz, 3H), 7.48 (d, J = 8 Hz, 2H), 7.08 (t, J = 10.8 Hz, 2H), 2.48 (s, 3H), 2.41 (s, 3H) , 2.35 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm) 179.27, 177.42, 153.50, 146.28, 145.41, 134.08, 130.65, 128.31, 128.12, 127.65, 125.94, 125.57, 116.68, 115.07, 97.00, 23.07, 21.65, 17.97; ESI-MS [M + Na]: 409.58 Da.

2.1.6.3 N′-(1-(7-hydroxy-4-methyl-2-oxo-2H-chromen-3-yl)ethylidene) isonicotinohydrazide ***(24c):*** Orange colour solid, yield (78%), M.P: 212–214 °C, FT-IR (KBr) $\bar{\nu}$/cm^{-1}: 3444, 3056, 2641, 1695, 1559; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 15.15 (s, 1H), 10.41 (s, 1H), 8.80 (d, J = 8, 2H), 7.85 (d, J = 7.2 Hz, 2H), 7.89 (t, J = 8 Hz, 1H), 7.47 (t, J = 8.8 Hz, 2H), 7.11 (m, 2H), 2.48 (s, 3H), 2.73 (s, 3H), 2.48 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 176.58, 154.55, 146.53, 144.48, 135.28, 131.62, 129.35, 128.92, 126.95, 126.35, 124.27, 117.48, 114.15, 98.09, 22.68, 18.91; ESI-MS [M + Na]: 360.72 Da.

2.2. Anticancer evaluation

2.2.1. Cell culture

A549 derived from human lung adenocarcinoma cells, HeLa derived from human cervical cancer cells, SK-N-SH derived from human neuroblastoma cells, MCF7 derived from human mammary gland adenocarcinoma cells and NRK-49F derived from normal rat kidney cells were procured from National Centre for Cell Science (Pune, India), maintained in DMEM media supplemented with 10% FBS, 0.1 mM non-essential aminoacids, 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics (60 µg/mL penicillin, 100 µg/mL streptomycin) at 37 °C in a 5% CO_2 humidified incubator with a subculture period of 2–3 days.

2.2.2. Preparation of samples for MTT assay

All the test compounds were taken in DMSO and further dilutions were made with sterile PBS (1×) to get desired concentrations of 10 mg/mL. All formulations were filtered through 0.22 µm sterile filter and 20 min of UV radiation before adding to the 96 well plates containing cells [27].

2.2.3. Cytotoxicity evaluation (MTT assay)

All the synthesized compounds were screened for *in vitro* cytotoxic activity against a panel of human tumour cell lines along with normal rat kidney cells (NRK 49F). The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells into purple formazan crystals which gets dissolved in DMSO and measured at 570 nm. Briefly, 5×10^3 exponentially growing cells were seeded into each 96 well plate (counted by Trypan blue exclusion dye method) allowed to grow till 60–70%, then compounds were added to the culture medium with the final concentrations ranging from 0.1, 1, 5 and 10 µM/mL and along with controls {negative (without compound) and positive (Doxorubicin)} incubated for 24 hoursin CO_2 incubator at 37 °C with a 90% humidified atmosphere and 5% CO_2. Then the media of the wells were replaced with 90 µl of fresh serum-free media and 10 µl of MTT (5 mg/mL of PBS), plates were incubated at 37 °C for 2 hrs, there after the above media was discarded and dried. 100 µl of DMSO was added in each well at 37 °C for 5 mins continuous added. The purple formazan crystals were dissolved and absorbance was immediately measured at 570 nm using Spectra Max plus 384 UV–Visible plate reader (Molecular Devices, Sunnyvale, CA, USA). IC_{50} values were determined by regression analysis software package of MS-excel.

2.3. In vitro tubulin polymerization inhibition assay

Tubulin was purified from goat brain by repeated cycles of polymerization and depolymerisation initially in PEM buffer [100 mM PIPES, 1 mM $MgSO_4$, 2 mM EGTA and 1 mM GTP] containing 50% glycerol and later in GEM [1 M sodium glutamate with pH 6.9, 1 mM EGTA and 1 mM $MgSO_4$] buffer. The crude protein homogenate was

obtained from minced and homogenised goat brain tissue. The homogenate was polymerised at 37 °C, pH = 6.9 with 1 mM GTP. The polymerised microtubules were sedimented with 151240 g at 35 °C. The sedimented microtubules were redissolved in ice cold PEM. The process was repeated four times to obtain the purified tubulin. Further, the tubulin protein was mixed with different concentrations of compounds (**12a** and **18a**) in PEM buffer (pH = 6.9), DMSO (8%), GTP (1 mM). Colchicine that inhibits microtubule polymerisation was used as positive control at different concentrations for the inhibition assay. Microtubule polymerisation was performed at 37 °C. The polymerised microtubules were sedimented with 25200 g at 37 °C for 30 mins. The concentration of free tubulin in the supernatant was measured by Bradford assay [28].

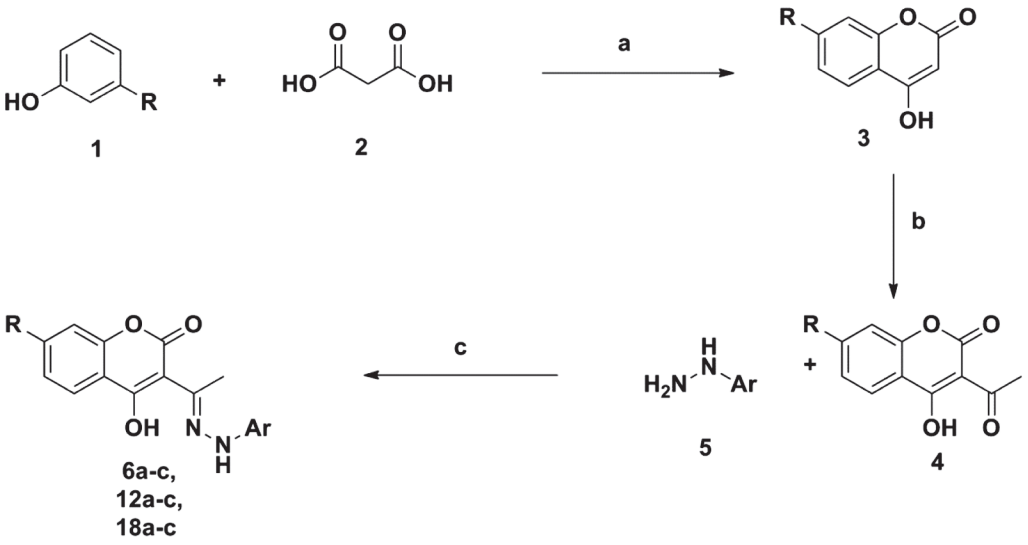
2.4. Computational methodology

2.4.1. Molecular docking simulations

The protein for the present investigation is tubulin which was retrieved from the Protein Data Bank (PDB) with the code 4YJ3 [10]. Protein preparation was performed by removing all the ligands, heteroatoms and water molecules followed by addition of missing hydrogens [29,30]. To make the protein ready for docking, the energy minimization was conducted until satisfied convergence gradient was obtained and then active site was selected around the substrate. Coumarin derivatives were drawn with the help of Chemdraw 3D software. Further, energy minimization done with CHARMm force field and was imported into the DSv16.1.0 [31]. The docking studies were performed with the CDOCKER, available in the Discovery studio software, for analyzing the binding affinities between the ligand and the protein. The prepared protein and ligands were imported into the DSv16.1.0 and the docking protocol was executed [32–34]. The docking results were studied with -CDOCKER interaction energy, hydrogen bond interaction and the binding mode pattern.

2.4.2. ADMET and drug-likeness studies

The compounds were evaluated for their drug-likeness properties



Reagents and conditions: a) $POCl_3/ZnCl_2$, 24h, 70 °C; b) $CH_3COOH/POCl_3$, 3h, 140 °C; c)EtOH/CH_3COOH, 3h, 60 °C.

6a; R=CH_3, Ar= Benzoyl

6b; R=CH_3, Ar= *p*-Tosyl

6c; R=CH_3, Ar=4-pyridinoyl

12a; R=OH, Ar= Benzoyl

12b; R=OH Ar= *p*-Tosyl

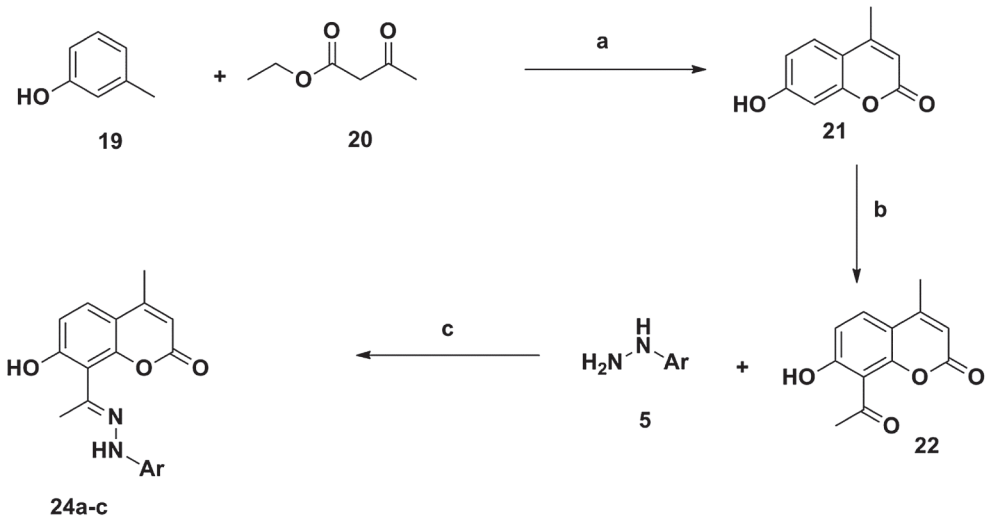
12c; R=OH, Ar= 4-Pyridinoyl

18a; R=H, Ar= Benzoyl

18b; R=H Ar= *p*-Tosyl

18c; R=H, Ar= 4-Pyridinoyl

Scheme 1.



Reagents and conditions: a) H_2SO_4, 2.5h, 10 °C; b) $CH_3COOH/POCl_3$, 3h, 140 °C; c)EtOH/CH_3COOH, 3h, 60 °C.

24a; Ar= Benzoyl

24b; Ar= *p*-Tosyl

24c; Ar= 4-Pyridinoyl

Scheme 2.

and pharmacokinetic properties which are retrieved by using the Osiris property explorer [35] and predicted by using ADMET server [36,37].

3. Results and discussion

3.1. Chemistry

The synthetic protocols of the target compounds were summarised in Scheme 1 and 2.

Scheme 1: (***6a–c, 12a–c and 18a–c***) compounds were synthesized from phenol and it's other derivatives (**3**) as a precursor, which was prepared by condensation of ***1*** and malonic acid. The formed coumarin (3) was further acylated with acetic acid in the presence of phosphorus oxychloride. Acylatedcoumarin (**4**) was further reacted with three different aromatic hydrazides to afford the final targeted compounds (Scheme 1).

Scheme 2: ***24a–c*** compounds were synthesized from 7-hydroxy 4-methyl coumarin as a precursor, which was prepared from m-cresol and ethyl acetoacetate in the presence of sulphuric acid. Further, the formed coumarin (**21**) was acylated by using acetic acid in the presence of

Table 1
In vitro cytotoxicity of target compounds against A549, HeLa, SKNSH, MCF-7 and NRK 49F cells.

S. No.	Sample	IC_{50} (in μMol)				
		A549	HeLa	SKNSH	MCF7	NRK 49F
1	**6a**	33.94 ± 0.34	29.97 ± 0.42	28.43 ± 0.24	28.49 ± 0.09	45.44 ± 1.18
2	**6b**	21.47 ± 0.18	60.45 ± 0.84	42.96 ± 0.12	29.06 ± 0.34	40.86 ± 1.15
3	**6c**	40.72 ± 0.27	38.17 ± 0.45	22.08 ± 0.61	31.12 ± 0.46	46.11 ± 2.36
4	**12a**	8.83 ± 0. 82	6.07 ± 0.12	8.24 ± 0.03	7.79 ± 0.41	89.53 ± 1. 82
5	**12b**	16.55 ± 0.86	14.17 ± 0.31	17.79 ± 0.16	12.31 ± 1.12	133.75 ± 1.16
6	**12c**	19.35 ± 0.12	13.98 ± 0.18	9.08 ± 0.92	14.81 ± 1.04	44.27 ± 0.48
7	**18a**	7.78 ± 0.14	12.36 ± 0.38	13.59 ± 0.23	11.93 ± 0.98	186.56 ± 1.68
8	**18b**	12.47 ± 0.32	15.28 ± 0.12	21.58 ± 0.16	12.12 ± 0.34	47.26 ± 0.16
9	**18c**	15.08 ± 0.42	13.02 ± 0.64	18.45 ± 0.85	16.26 ± 0.16	52.85 ± 0.24
10	**24a**	25.49 ± 0.46	39.13 ± 0.34	26.98 ± 0.18	28.73 ± 1.23	43.05 ± 0.36
11	**24b**	29.35 ± 0.18	35.022 ± 0.09	31.83 ± 0.62	32.66 ± 0.86	47.47 ± 1.21
12	**24c**	21.76 ± 0.28	28.41 ± 0.16	31.77 ± 0.54	35.59 ± 1.68	45.79 ± 0.46
13	**Dox**	6. 22 ± 0.03	7.01 ± 0.015	6.42 ± 0.56	9.86 ± 0.12	14.22 ± 0.18

IC_{50} is the 50% inhibitory concentration of the samples, and the results were represented as average values ± standard deviation.
A-549, human lung adenocarcinoma cells: HeLa, human cervical cancer cells: SK-N-SH, human neuroblastoma cells: MCF-7, mammary gland adenocarcinoma cells and NRK-49F, normal rat kidney cells.
Dox = Doxorubicin used as positive control (Standard).

Table 2
Tubulin polymerisation inhibition assay IC_{50} (μM).

Sample	IC_{50} (μM)
12a	14.25 ± 1.25
18a	16.96 ± 0.92
Colchicine	7.35 ± 1.05

phosphorus oxychloride. Acylatedcoumarin (**22**) was finally reacted with three different aromatic hydrazides to afford the final compounds (Scheme 2).

3.2. Antiproliferative assay

We have evaluated all the synthesized coumarin derivatives for the screening of anticancer activity using MTT assay against four human cancer cell lines **A549** derived from human lung adenocarcinoma cells, **HeLa** derived from human cervical cancer cells, **SK-N-SH** derived from human neuroblastoma cells, **MCF-7** derived from mammary gland adenocarcinoma cells and **NRK-49F** derived from normal rat kidney cells using doxorubicin as standard drug. Based on the screening results, we observed that these compounds exhibited good to moderate cytotoxicity with IC_{50} values ranging from **6.07** to **60.45 μM** against all the tested cancer cell lines. The results suggested the compounds **12a** and **18a** are promising antiproliferative agents due to their functional group diversity. The cytotoxicity results are shown in Table 1.

3.3. Tubulin polymerisation inhibition assay

Many of the chemotherapeutic agents used in cancer today target tubulin. By targeting tubulin they cause mitotic arrest and thus are termed as antimitotic drugs. These drugs attack the cancer cells at both primary and metastatic phase. These agents primarily bind to the beta-tubulin protein in the mitotic spindle. The most potent compounds (**12a and 18a**) find in MTT assay were allowed for tubulin polymerisation inhibition assay. The compounds showed good inhibitory activity compared with standard antitubulin drug colchicine. The IC_{50} values of compounds **12a** and **18a** are 14.25 and 16.96 μM respectively (see Table 2).

3.4. Molecular docking simulation

Molecular docking studies were carried out to identify the interactions between compounds and target protein. Discovery studio software was used for the docking simulation where the ligands were docked into the protein to optimize the scoring function. The crystal structure of the tubulin protein (**PDB; 4YJ3**) was obtained from the PDB (www.rcsb.org/pdb). Both the compounds **12a** and **18a** were docked with the target protein. The docking studies on protein revealed that these ligands showed good covalent cross-linkage mode of binding with the tubulin. Each compound showed three strong conventional hydrogen bond interactions. Fig. 1 depicted the interactions of **12a** ligand with protein exhibiting hydrogen bonds with **Asn-101, Thr-179 and ASN-228** amino acid residues. Fig. 2 depicted the interactions of **18a** ligand with protein. The compound exhibited hydrogen bonds with **GLN-11,**

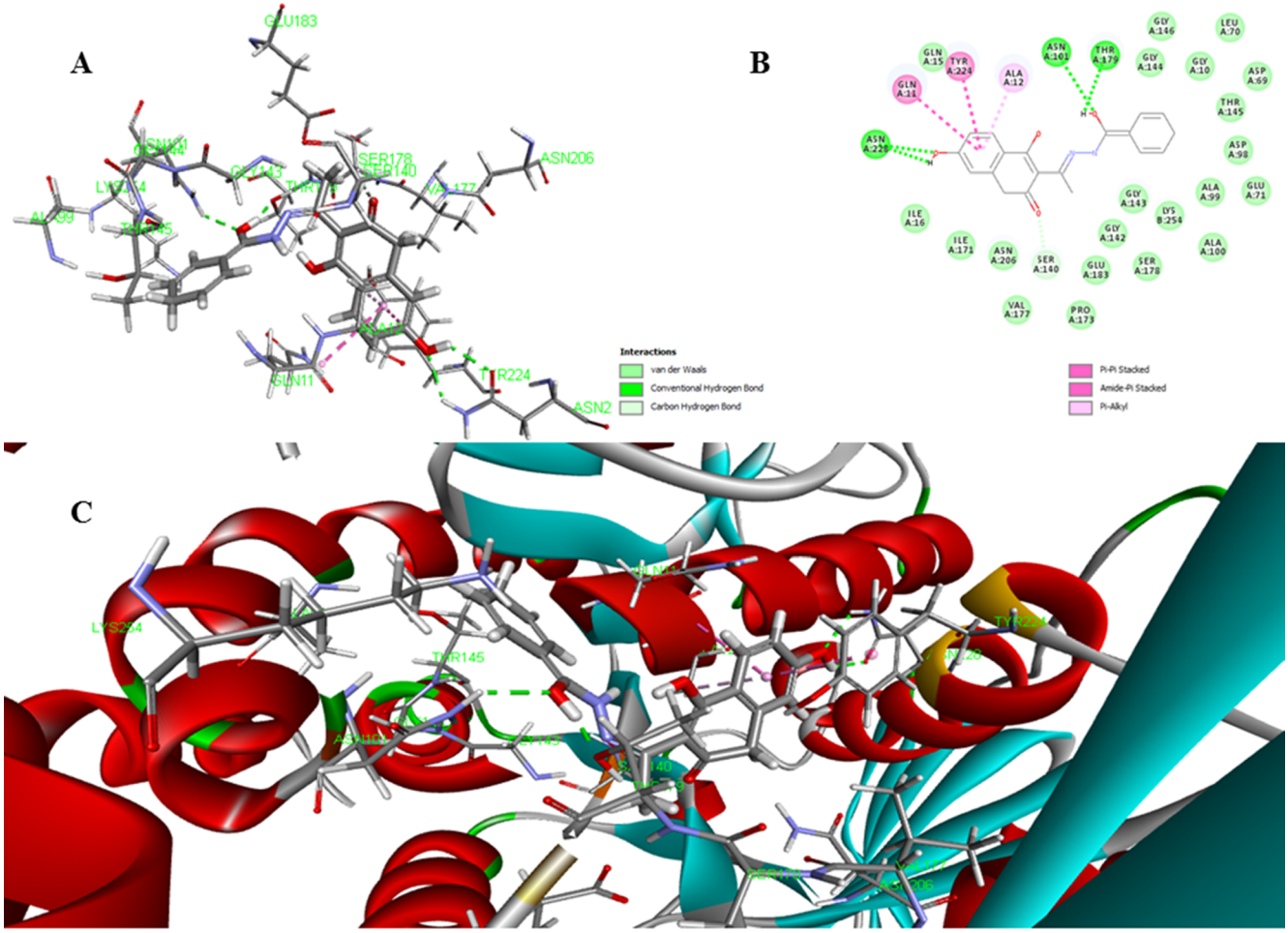


Fig. 1. Plausible binding mode of the compound 12a with tubulin protein (A) stick model interactions with tubulin protein (B) 2D representation interactions (C) 3D view of proposed binding mode.

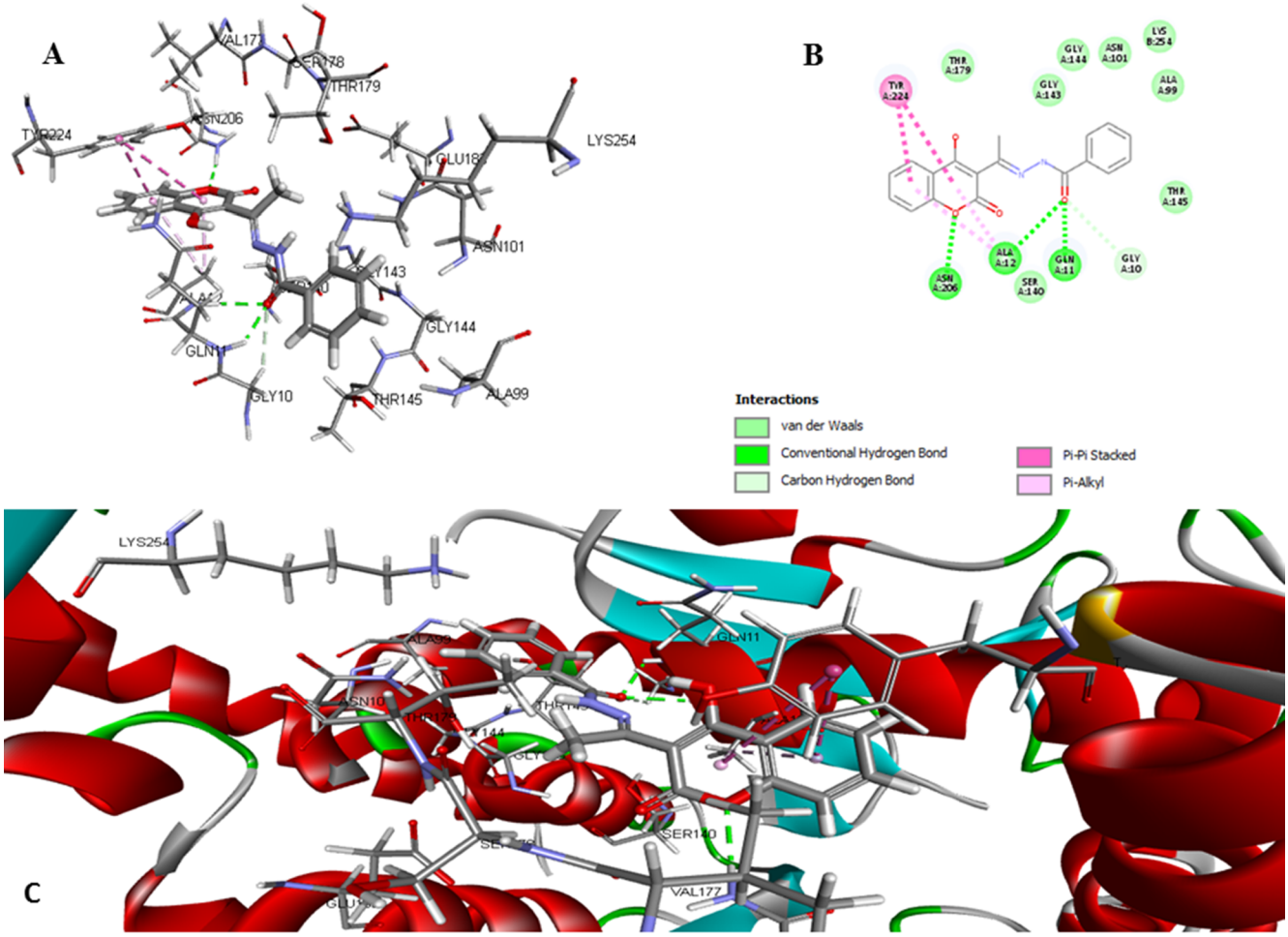


Fig. 2. Plausible binding mode of the compound **18a** with tubulin protein (A) stick model interactions with tubulin protein (B) 2D representation interactions (C) 3D view of proposed binding mode.

Table 3
Molecular properties (Lipinski Rule). (Results were predicted by using the Osiris property explorer software available at http://www.organic-chemistry.org/prog/peo/.)

Sample	MW	Log P	HBA	HBD
6a	336.34	3.35	5	2
6b	386.42	2.77	6	2
6c	337.33	2.35	6	2
12a	338.31	2.66	6	3
12b	388.39	2.08	7	3
12c	339.30	1.66	7	3
18a	332.31	3.01	5	2
18b	372.40	2.43	6	2
18c	323.30	2.01	6	2
24a	336.34	3.127	5	2
24b	386.42	2.54	6	2
24c	337.33	2.12	6	2

MW = Molecular weight, LogP = Partition coefficient, HBA = Hydrogen bond acceptors, HBD = Hydrogen bond donors.

ALA-12, and ASN-206 amino acid residues.

3.5. Physicochemical properties

To evaluate the drug-like properties we used *Lipinski's rule of five*, according to this rule the orally bioavailable drug should have molecular weight (MW) should be less than 500 Daltons, partition coefficient (LogP) should not greater than 5, hydrogen bond acceptors (HBA) are not more than 10 and hydrogen bond donors (HBD) are not more than 5. The results were predicted by using Osiris property explorer software [35]. The results demonstrated that all the compounds obey the Lipinski rule and showed good properties (Table 3).

3.6. Pharmacokinetic properties

The evaluation of pharmacokinetic properties is probably the most important aspect to develop a novel drug molecule. The drug molecule requires good pharmacokinetic properties along with medicinal properties. To determine the pharmacokinetic behavior of our synthesized compounds PreADMET server was employed. The general evaluation in ADMET, absorption properties such as human intestinal absorption (HIA), CaCo-2 permeability, MDCK cell permeability, skin permeability (log kp) results indicate that the compounds are showing good absorption properties to cross the gut wall by passive diffusion mechanism to reach the target. In a general sense the drugs are majorly distributed through plasma proteins. However some drugs can distribute through blood brain barrier (BBB). The distribution properties of our compounds suggest that these are distributed through plasma proteins only and there is no ability to cross BBB (Table 4). The compounds metabolic properties were predicted by the behavior of cytochrome P450 and their isoform enzymes. Other than CYP_2C9, CYP_2C9 isoforms all other isoforms of CYP450 are not showing any competitive inhibitory or substrate activity with the all synthesized compounds (Table 5).

Table 4
Absorption and distribution properties of the synthesized compounds (Results were predicted by using the server https://preadmet.bmdrc.kr/adme.)

Sample	Absorption				Distribution	
	Human intestinal absorption (HIA %)	CaCo-2 cell permeability (nm/sec)	MDCK cell permeability (nm/sec)	Skin permeability (logkp, cm/hour)	Plasma Protein Binding (%)	Blood-Brain Barrier penetration (c.brain/c.blood)
6a	93.89	16.06	9.27	−3.27	81.79	0.23
6b	96.78	12.48	0.05	−1.45	88.30	0.098
6c	93.65	12.23	37.78	−3.90	68.03	0.04
12a	89.47	15.59	35.70	−4.19	76.57	0.11
12b	90.10	0.38	0.63	−3.02	75.75	0.05
12c	85.99	11.25	23.47	−4.67	59.99	0.02
18a	93.77	14.93	34.44	−3.40	78.31	0.18
18b	94.30	0.52	2.24	−2.90	76.85	0.44
18c	93.22	10.21	19.67	−4.01	62.42	0.048
24a	93.89	18.97	3.08	−3.20	84.50	0.125
24b	94.40	0.64	0.081	−2.67	89.50	0.04
24c	93.66	16.86	23.23	−3.83	74.71	0.018

Table 5
Metabolism of all synthesized compounds via hepatic microsomal isoforms. (Results were predicted by using the server https://preadmet.bmdrc.kr/adme.)

Sample	CYP_2C9 Inhibition	CYP_2C9 inhibition	CYP_2D6 inhibition	CYP_2D6 substrate	CYP_3A4 inhibition	CYP_3A4 substrate	Pgp_inhibition
6a	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
6b	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Weak-inhibitor	Inhibitor
6c	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
12a	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
12b	Inhibitor	Inhibitor	Non-inhibitor	Non-Inhibitor	Non-inhibitor	Weak-Inhibitor	Non-inhibitor
12c	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
18a	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
18b	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
18c	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
24a	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Weak-Inhibitor	Non-inhibitor
24b	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Weak-Inhibitor	Non-inhibitor
24c	Inhibitor	Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Weak-Inhibitor	Non-inhibitor

4. Structure activity relationship

From the results of *in vitro* antiproliferative assay and *in silico* evaluation studies the structural activity relationship (SAR) of the compounds was established for 4,7-dihydroxycoumarin and 4-hydroxyl coumarin derivatives which showed good cytotoxicity results, good drug-likeness as well as good pharmacokinetic properties. Three different hydrazine derivatives were substituted to the coumarin ring such as benzoyl, toulenesulphonyl and pyridinoyl aromatic rings. Among these three, the benzoyl derivatives showed best results when compared with the other two substitutions.

5. Conclusion

We have successfully synthesized different series of coumarin based hydrazone derivatives and these compounds were characterized by FT-IR, NMR and Mass spectroscopic methods. Furthermore, these compounds were screened for anticancer activity against A-549, Hela, SK-N-SH, MCF-7 human cancer cell lines by using MTT assay as well as against normal rat kidney cell line NRK-49F. All the compounds showed good to moderate cytotoxicity. Among all compounds, **12a** and **18a** exhibited potent activity against all tested cancer cell lines. Tubulin polymerisation inhibition assay was performed for the potent molecules **12a** and **18a** which showed good inhibition towards tubulin protein when compared with colchicine. Docking studies were performed to have an idea of the mechanism and binding modes of the new active compounds towards tubulin protein. The results revealed good interactions with target tubulin at the active sites and stable interactions with ligands. We have studied the drug-like properties and ADMET properties of the all compounds. In conclusion we propose that the compounds **12a** and **18a** might be used as lead molecules for further investigation.

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Declaration of Competing Interest

The authors declared that they have no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103143.

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