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Synthesis of coumarin analogs appended with quinoline and thiazole moiety and their apoptogenic role against murine ascitic carcinoma

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ABSTRACT

The synthesis and antiproliferative effect of a series of quinoline and thiazole containing coumarin analogs **12a-d** and **13a-f** respectively, on mice leukemic cells was performed. The chemical structures of newly synthesized compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis. The result indicates that, 7-methoxy-2-oxo-2H-chromene-3-carboxylic acid [4-(4-methoxy-phenyl)-thiazol-2-yl]-amide (**13f**) showed potent activity against EAC and DLA cells in MTT (15.3 μM), tryphan blue (15.6 μM) and LDH (14.2 μM) leak assay with 5-fluorouracil as a standard. Further, the anti-neoplastic effect of the compound **13f** was verified against Ehrlich ascites tumour by BrdU incorporation, TUNEL, FACS and DNA fragmentation assays. Experimental data showed that compound **13f** induces the apoptotic cell death by activating apoptotic factors such as caspase-8 &-3, CAD, Cleaved PARP, γ-H2AX and by degrading genomic DNA of cancer cells and thereby decreasing the ascitic tumour development in mice. Besides, compound **13f** was also subjected for docking studies to approve the *in vitro* and *in vivo* studies. The data revealed that the compound **13f** has very good interaction with caspase 3 protein by binding with amino acid Arg 207 through hydrogen bond.

1. Introduction

Cancer cells have progressed hallmark mechanisms to escape from death. One of the hallmarks in the advancement of cancer cells is an ability to overcome and acquire resistance to adverse conditions and thereby become immortal [1]. Cancer cells escapes from the apoptosis because of the disturbance of spatiotemporal behavior of signaling cascades in cancer cells and down regulation of tumour suppressor genes and over expression of oncogenes [2]. Impairment of this native defense mechanism of a cancer cell, promotes aberrant cellular proliferation and the accumulation of genetic defects, ultimately resulting in tumorigenesis, and frequently confers resistance towards the drug [3]. Many researchers have gained their interest in developing anticancer agents by killing the cancer cells, mainly mediated through the activation of apoptosis [4,5].

Coumarins are one such set of candidates for the apoptosis targeted therapy and it has gained much attention for its ability to preferentially kill cancer cells. Structurally, coumarins, also known as benzopyran-2-ones and it is known to be a good class of naturally occurring compounds that possess promising therapeutic perspectives. Due to diversity in their structural complexity, these, ranging from simple substituted coumarins to polysubstituted polycyclic/fused coumarins gained a great attention due to their wide range of biological properties [6–9], usually associated with low toxicity [10,11]. Furthermore, their cancer chemo preventive properties have been recently emphasized [6,12]. The apoptosis and differentiation induced activities of coumarins extend to several different cell line models *in vitro*, and they appear to be the most promising in terms of cancer treatment [13]. On the other hand, quinoline and thiazole derivatives play an important role in showing anticancer activity [14–17]. In the light of these facts, and as a continuation of our previous reported work [18–20], we planned to synthesize a novel series of coumarin analogs bearing a quinoline **12a-d** and thiazole moiety **13a-f**, in order to study their structure activity relationship and to screen for a potent apoptogen against carcinoma. These results were also further supported by *in silico* studies [21,22].

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2. Experimental

2.1. Chemistry

Chemicals were purchased from Sigma Aldrich Chemical Co. TLC was accomplished on aluminum-backed silica plates and visualized by UV-light. Melting points were recorded on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded on FT-IR Shimadzu 8300 spectrometer, ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker NMR spectrometer in DMSO-d_6 and the chemical shifts were recorded in parts per million downfield from tetramethylsilane. Mass spectra were obtained with a VG70-70H spectrometer. Elemental analysis was done by Perkin Elmer 2400 and the results were within 0.4% of the calculated value.

2.1.1. Synthesis of ethyl (quinolin-8-yloxy)-acetate (3)

Compound **3** [ethyl (quinolin-8-yloxy)-acetate] was obtained by refluxing the mixture of 8-hydroxy quinolone (**1,** 0.013 mol) and ethyl chloroacetate (**2**, 0.026 mol) in the presence of anhydrous potassium carbonate (0.019 mol) and dry acetone (50 mL) as solvent for 8 h. The reaction mixture was cooled and solvent was removed by distillation under vacuum. The residual mass was quenched with cold water to remove potassium carbonate, and extracted with ether (3 × 50 mL). The ether layer was washed with 10% sodium hydroxide solution (3 × 50 mL) followed by water (3 × 30 mL), dried over anhydrous sodium sulphate and evaporated to dryness to obtain crude solid, which, on recrystallization with ethanol afforded desired compound **3**[ethyl (quinolin-8-yloxy)-acetate] [23].

3: Yield 90%. mp 68–70 °C. FT-IR (KBr, cm^{-1}): 1760 (ester, C═O). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 1.2 (t, 3H, CH_3 of ester, J = 7.1 Hz), 4.1 (q, 2H, CH_2 of ester, J = 7.1 Hz), 4.9 (s, 2H, OCH_2), 6.9–7.8 (m, 6H, Ar-H). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 14.1, 61.3, 65.3, 107.2, 120.4, 121.8, 126.8, 129.3, 135.7, 138.8, 149.0, 155.4, 169.2. LC–MS m/z 232.1 (M + 1). Anal. Calcd. For $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.56; H, 5.69; N, 6.01%.

2.1.2. Synthesis of (quinolin-8-yloxy)-acetic hydrazide (4)

Hydrazine hydrate (0.1686 mol) was added to a solution of compound **3**, (0.0562 mol) in ethanol (100 mL) at room temperature and the reaction mixture was stirred for 2 h. A white solid was separated out, which was quenched with water, filtered and washed with water (3 × 50 mL). Finally, the solid was recrystallized with ethanol and dried under vacuum to obtain compound **4 [**(quinolin-8-yloxy)-acetic hydrazide] [24].

4: Yield 75%. mp 130–132 °C. FT-IR (KBr, cm^{-1}): 1730 (C═O). 3400–3500 (NH−NH_2). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 4.4 (bs, 2H, NH_2) 4.8 (s, 2H, OCH_2), 6.9–8.1 (m, 6H, Ar-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 68.7, 107.2, 120.4, 121.8, 126.8, 129.3, 135.7, 138.8, 149.0, 155.4, 166.3. LC–MS m/z 218.1 (M + 1). Anal. Calcd for $C_{11}H_{11}N_3O_2$: C, 60.82; H, 5.10; N, 19.34. Found: C, 60.77; H, 5.17; N, 19.37%.

2.1.3. General procedure for the synthesis of 2-amino-4-phenyl thiazoles (7a-b)

2-Amino-4-phenyl thiazoles **(7a-b)** were synthesized by using substituted acetophenones **(5a-b,** 0.0037 mol) and thiourea **(6,** 0.004 mol). The reaction mixture was refluxed for 16 h in the presence of iodine (0.0074 mol) and ethanol as a solvent. It was further treated with sodium hydroxide solution to get the products **7a-b,** which were recrystallized from ethanol to get needle like crystals [25].

7a: 2-Amino-4-phenyl thiazole: Yield 90%. mp 148–149 °C. FT-IR (KBr, cm^{-1}): 1698 (C═N), 3370–3395 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 6.8–7.6 (m, 6H, Ar-H), 7.8 (bs, 2H, NH_2). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 84.0, 126.4, 128.0, 128.7, 134.3, 157.0, 178.2. LC–MS m/z 177 (M + 1). Anal. Calcd for $C_9H_8N_2S$: C, 61.33; H, 4.58; N, 15.90; S, 18.19. Found: C, 61.26; H, 4.51; N, 15.98; S, 18.11%.

7b: 4-(4-Methoxy phenyl) thiazol-2-amine: Yield 85%. mp 168–170 °C. FT-IR (KBr, cm^{-1}): 1698 (C═N), 3340–3370 (N−H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 3.5 (s, 3H, OCH_3), 6.8–7.6 (m, 5H, Ar-H), 7.8 (bs, 2H, NH_2). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 24.3, 84.0, 126.3, 129.0, 131.3, 137.6, 157.0, 178.2. LC–MS m/z 207 (M + 1). Anal. Calcd for $C_{10}H_{10}N_2OS$: C, 58.23; H, 4.89; N, 13.58; S, 15.55. Found: C, 58.28; H, 4.81; N, 13.52; S, 15.48%.

2.1.4. General procedure for the synthesis of ethyl 2-oxo-2H-chromene-3-carboxylates (10a-d)

Substituted salicylaldehydes **(8a-d,** 0.045 mol) and diethylmalonate **(9,** 0.045 mol) were dissolved in ethanol (150 mL) to get a clear solution. Subsequently, by adding 1–2 drops of piperidine was added to the reaction mixture was refluxed for 5 h. After the completion of the reaction (monitored by thin layer chromatography in toluene : ethyl acetate solvent system), the content was concentrated to a small volume. Then the reaction mixture was poured into crushed ice and the resulted solid was filtered, dried and recrystallized from ethanol to afford desired compounds (**10a-d**) [ethyl 2-oxo-2H-chromene-3-carboxylates] [26].

10a: Ethyl 2-oxo-8-methyl 2H-chromene-3-carboxylate: Yield: 90%, mp 124–125 °C. FT-IR (KBr, cm^{-1}): 1675 (C═O), 1745 (ester, C═O).^{1}H NMR (400 MHz) (DMSO-d_6): δ 1.2 (t, 3H, CH_3 of ester, J = 7.1 Hz), 3.5 (q, 2H, CH_2 of ester, J = 7.1 Hz), 7.2–8.2 (m, 4H, Ar–H), 9.1 (s, 1H, coumarin ring-H). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 14.2, 61.4, 113.8, 121.5, 122.2, 125.5, 126.8, 128.4, 139.7, 150.1, 159.4, 165.0. LC–MS m/z 219.0 (M + 1). Anal. Calcd. for $C_{12}H_{10}O_4$: C, 66.05; H, 4.62;. Found: C, 66.12; H, 4.69%.

10b: Ethyl 8-methyl-2-oxo- 2H-chromene-3-carboxylate: Yield: 92%, mp 136–138 °C. FT-IR (KBr, cm^{-1}): 1655 (C═O), 1740 (ester, C═O). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 1.2 (t, 3H, CH_3 of ester, J = 7.1 Hz), 2.3 (s, 3H, Ar − CH_3), 3.5 (q, 2H, CH_2 of ester, J = 7.1 Hz), 7.2–8.2 (m, 3H, Ar–H), 9.1 (s, 1H, coumarin ring-H). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 14.2, 18.7, 61.4, 113.8, 122.1, 123.8, 125.4, 128.6, 131.7, 139.7, 150.4, 159.4, 165.0. LC–MS m/z 233.2 (M + 1). Anal. Calcd. for $C_{13}H_{12}O_4$: C, 67.23; H, 5.21;. Found: C, 67.17; H, 5.30%.

10c: Ethyl 7-fluoro-2-oxo- 2H-chromene-3-carboxylate: Yield: 89%, mp 140–142 °C. FT-IR (KBr, cm^{-1}): 1660 (C═O), 1740 (ester, C═O). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 1.2 (t, 3H, CH_3 of ester, J = 7.1 Hz), 3.5 (q, 2H, CH_2 of ester, J = 7.1 Hz), 7.2–8.2 (m, 3H, Ar–H), 9.1 (s, 1H, coumarin ring-H). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 14.2, 61.4, 109.6, 112.2, 113.8, 117.8, 128.4, 139.7, 151.7, 159.4, 162.5, 165.0.LC–MS m/z 237.0 (M + 1). Anal. Calcd. for $C_{12}H_9FO_4$: C, 61.05; H, 3.82;. Found: C, 61.12; H, 3.88%.

10d: Ethyl 7-methoxy-2-oxo- 2H-chromene-3-carboxylate: Yield: 93%, mp 151–153 °C. FT-IR (KBr, cm^{-1}): 1665 (C═O), 1750 (ester, C═O). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 1.2 (t, 3H, CH_3 of ester, J = 7.1 Hz), 3.5 (q, 2H, CH_2 of ester, J = 7.1 Hz), 3.7 (s, 3H, OCH_3), 7.2–8.2 (m, 3H, Ar–H), 9.1 (s, 1H, coumarin ring-H). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 14.2, 55.8, 61.4, 113.8, 113.9, 119.1, 123.2, 126.5, 139.7, 141.8, 157.0, 159.4, 165.0.LC–MS m/z 249.2 (M + 1). Anal. Calcd. for $C_{13}H_{12}O_5$: C, 62.90; H, 4.87;. Found: C, 62.98; H, 4.81%.

2.1.5. General procedure for the synthesis of 2-oxo-2H-chromene-3-carboxylic acids (11a-d)

Compounds (**10a-d) [**ethyl 2-oxo-2H-chromene-3-carboxylates] (6.0 mmol) were dissolved in ethanol (15 mL) and treated with a 5 mL of 15 mmol solution of sodium hydroxide solution. The reaction mixture was refluxed for 6 h, cooled and acidified with 1 N hydrochloric acid. The precipitate so obtained was filtered, washed with water and finally recrystallized from methanol to afford desired compounds (**11a-d**) with good yields [27].

11a: 2-oxo-2H-chromene-3-carboxylic acid: Yield: 85%. mp

189–192 °C. FT-IR (KBr, cm^{-1}): 1665 (C═O). 1730 (acid, C═O), 3330–3380 (OH). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 6.8–8.2 (m, 4H, Ar–H), 9.1 (s, 1H, coumarin ring-H), 13.2 (s, 1H, COOH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 113.1, 121.5, 122.2, 125.5, 126.8, 128.4, 142.5, 150.1, 159.4, 166.2.LC–MS *m/z* 191.1 (M + 1). Anal. Calcd. For $C_{10}H_6O_4$: C, 63.16; H, 3.18. Found: C, 63.10; H, 3.10%.

11b: 8-Methyl-2-oxo-2H-chromene-3-carboxylic acid: Yield: 84%. mp 199–200 °C. FT-IR (KBr, cm^{-1}): 1660 (C═O). 1725 (acid, C═O), 3325–3375 (OH). ^{1}H NMR (400 MHz) (DMSO-d_6): δ2.3 (s, 3H, Ar − CH_3), 6.8–8.2 (m, 3H, Ar–H), 9.1 (s, 1H, coumarin ring-H), 13.2 (s, 1H, COOH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 18.7, 113.1, 122.1, 123.8, 125.4, 128.6, 131.7, 142.5, 150.4, 159.4, 166.2.LC–MS *m/z* 205.0 (M + 1). Anal. Calcd. For $C_{11}H_8O_4$: C, 64.70; H, 3.95. Found: C, 64.79; H, 3.90%.

11c: 7-Fluoro-2-oxo-2H-chromene-3-carboxylic acid: Yield: 89%. mp 212–223 °C. FT-IR (KBr, cm^{-1}): 1660 (C═O). 1735 (acid, C═O), 3320–3370 (OH), ^{1}H NMR (400 MHz) (DMSO-d_6): δ 6.8–8.2 (m, 3H, Ar–H), 9.1 (s, 1H, coumarin ring-H), 13.2 (s, 1H, COOH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 109.6, 112.2, 113.1, 117.8, 128.4, 142.5, 151.7, 159.4, 162.5, 166.2. LC–MS *m/z* 209.1 (M + 1). Anal. Calcd. For $C_{10}H_5FO_4$: C, 57.70; H, 2.42. Found: C, 57.62; H, 2.48%.

11d: 7-Methoxy-2-oxo-2H-chromene-3-carboxylic acid: Yield: 91%. mp 184–186 °C. FT-IR (KBr, cm^{-1}): 1655 (C═O), 1715 (acid, C═O), 3320–3370 (OH).^{1}H NMR (400 MHz) (DMSO-d_6): δ3.7 (s, 3H, OCH_3), 6.8–8.2 (m, 3H, Ar–H), 9.1 (s, 1H, coumarin ring-H), 13.2 (s, 1H, COOH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 55.8, 113.1, 113.9, 119.1, 123.2, 126.5, 141.8, 142.5, 157.0, 159.4, 166.2. LC–MS *m/z* 221.0 (M + 1). Anal. Calcd. For $C_{11}H_8O_5$: C, 60.00; H, 3.66. Found: C, 60.10; H, 3.70%.

*2.1.6. General procedure for the synthesis of 2-oxo-2H-chromene-3-carboxylic acid N'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide analogs (**12a-d**)*

To compounds [2-oxo-2H-chromene-3-carboxylic acid] (**11a-d,** 0.0037 mol) in dry dichloromethane (15 mL), lutidine (1.2 vol.) was added at 25–30 °C, followed by the addition of compound (**4,** 0.0037 mol) and the mixture was stirred at 25–30 °C for 30 min. The reaction was cooled to 0–5 °C and TBTU (0.0037 mol) was added over a period of 30 min while maintaining the temperature below 5 °C. The reaction was stirred overnight and monitored by TLC using chloroform: methanol (9:1) as an eluent. The reaction mixture was diluted with 20 mL of dichloromethane and washed with 1.5 N hydrochloric acid (20 mL). The organic layer was washed with water (3 × 25 mL), dried over anhydrous sodium sulphate, concentrated to a syrupy liquid and recrystallized twice from diethyl ether to afford compounds **12a-d** in good yields.

12a: 2-Oxo-2H-chromene-3-carboxylic acid *N*'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide: Yield: 85%. mp 180–184 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3100–3205 (NH-NH). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 5.0 (s, 2H, OCH_2), 7.2–8.6 (m, 10H, Ar–H), 9.08 (s, 1H, coumarin ring-H), 11.0 (s, 1H, −NH−CO) 12.3 (s, 1H, −NH of $COCH_2$). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 68.7, 107.2, 114.1, 120.4, 121.5, 121.8, 122.2, 125.5, 126.8, 128.4, 129.3, 135.7, 138.5, 138.8, 149.0, 150.1, 155.4, 159.4, 166.3, 165.9, 166.4. LC–MS: *m/z* 390.4 (M + 1). Anal. Calcd. For $C_{21}H_{15}N_3O_5$: C, 64.78; H, 3.88; N, 10.79. Found: C, 64.71; H, 3.81; N, 10.71%.

12b: 8-Methyl-2-oxo-2H-chromene-3-carboxylic acid *N*'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide: Yield: 86%. mp 120–122 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3110–3215 (NH-NH). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 2.3 (s, 3H, Ar − CH_3), 5.0 (s, 2H, OCH_2), 7.0–8.6 (m, 9H, Ar–H), 9.10 (s, 1H, coumarin ring-H), 11.09 (s, 1H, -NH − CO) 12.36 (s, 1H, -NH of $COCH_2$). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 18.7, 68.7, 107.2, 114.1, 120.4, 120.6, 121.8, 122.1, 123.8, 125.4, 126.8, 129.3, 131.7, 135.7, 138.5, 138.8, 149.0, 150.4, 155.4, 159.4, 166.3. 165.9.LC–MS: *m/z* 404.1 (M + 1). Anal. Calcd. For $C_{22}H_{17}N_3O_5$: C, 65.50; H, 4.25; N, 10.42. Found: C, 65.51; H, 4.21; N, 10.38%.

12c: 7-Fluoro-2-oxo-2H-chromene-3-carboxylic acid *N*'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide: Yield: 80%. mp 182–184 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3115–3220 (NH-NH). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 5.0 (s, 2H, OCH_2), 7.2–8.6 (m, 9H, Ar–H), 9.10 (s, 1H, coumarin ring-H), 11.09 (s, 1H, -NH − CO) 12.36 (s, 1H, -NH of $COCH_2$). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 68.7, 107.2, 109.6, 112.2, 114.1, 117.8, 120.4, 121.8, 126.8, 128.4, 129.3, 135.7, 138.5, 138.8, 149.0, 151.7, 155.4, 159.4, 162.5, 165.9, 166.3. LC–MS: *m/z* 408.1 (M + 1). Anal. Calcd. For $C_{21}H_{14}FN_3O_5$: C, 61.92; H, 3.46; N, 10.32. Found: C, 61.97; H, 3.49; N, 10.35%.

12d: 7-Methoxy-2-oxo-2H-chromene-3-carboxylic acid *N*'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide: Yield: 87%. mp 190–192 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3105–3210 (NH-NH). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 3.7 (s, 3H, OCH_3), 5.0 (s, 2H, OCH_2), 7.2–8.2 (m, 9H, Ar–H), 9.10 (s, 1H, coumarin ring-H), 11.09 (s, 1H, -NH − CO) 12.36 (s, 1H, -NH of $COCH_2$). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 55.8, 68.7, 107.2, 107.6, 111.0, 114.1, 114.5, 120.4, 121.8, 126.8, 127.8, 129.3, 135.7, 138.5, 138.8, 149.0, 151.1, 155.4, 159.4, 160.2, 165.9, 166.3. LC–MS: *m/z* 420.3 (M + 1). Anal. Calcd. For $C_{22}H_{17}N_3O_6$: C, 63.01; H, 4.09; N, 10.02. Found: C, 63.07; H, 4.02; N, 10.08%.

*2.1.7. General procedure for the synthesis of 2-oxo-2H-chromene-3-carboxylic acid (4-phenyl-thiazol-2-yl)-amide analogs (**13a-f**)*

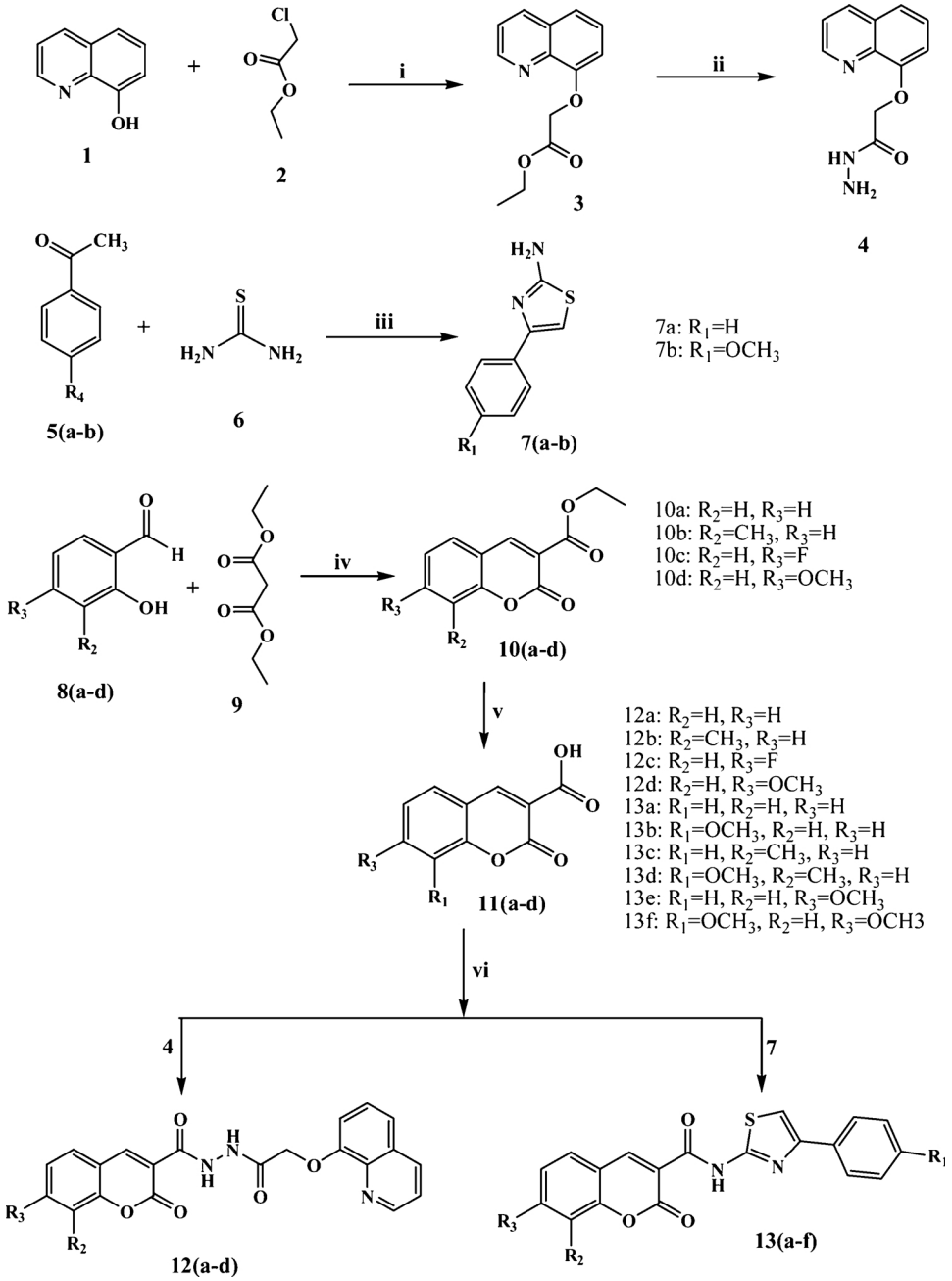
To compounds [2-oxo-2H-chromene-3-carboxylic acids] (**11a–d,** 0.0037 mol) in dry dichloromethane (15 mL), lutidine (1.2 vol.) was added at 25–30 °C, followed by the addition of compound (**7a-b,** 0.0037 mol) and the mixture was stirred at 25–30 °C for 30 min. The reaction was cooled to 0–5 °C and TBTU (0.0037 mol) was added over a period of 30 min while maintaining the temperature below 5 °C. The reaction was stirred overnight and the completion of the reaction was monitored by TLC using chloroform:methanol (9:1) as an eluent. The reaction mixture was diluted with 20 mL of dichloromethane and washed with 1.5 N hydrochloric acid (20 mL). Then the organic layer was washed with water (3 × 25 mL), dried over anhydrous sodium sulphate, concentrated to a syrupy liquid and recrystallized twice from diethyl ether to afford compounds **13a-f** in good yields.

13a: 2-Oxo-2H-chromene-3-carboxylic acid (4-phenyl-thiazol-2-yl)-amide: Yield: 87%. mp 202–204 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3145–3185 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 7.0–7.8 (m, 10H, Ar–H), 8.3 (s, 1H, coumarin ring-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 100.0, 114.2, 121.5, 122.3, 125.5, 126.8, 127.5, 128.4, 128.8, 129.3, 133.1, 138.6, 148.2, 150.2, 159.5, 163.1, 164.3. LC–MS *m/z* 349.1 (M + 1). Anal. Calcd. For $C_{19}H_{12}N_2O_3S$: C, 65.50; H, 3.47; N, 8.04; S, 9.20. Found: C, 65.56; H, 3.51; N, 8.01; S, 9.26%.

13b: 2-Oxo-2H-chromene-3-carboxylic acid [4-(4-methoxy-phenyl)-thiazol-2-yl]-amide: Yield: 82%. mp 185–187 °C. FT-IR (KBr, cm^{-1}): 1635 (C═O), 1675 (C═O, amide), 3125–3165 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 3.7 (s, 3H, OCH_3), 7.0–7.8 (m, 9H, Ar–H), 8.3 (s, 1H, coumarin ring-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 55.8, 99.9, 114.1, 114.8, 121.5, 122.2, 125.5, 126.8, 128.5, 138.5, 148.1, 150.1, 159.4, 160.6, 163.0, 164.2. LC–MS *m/z* 379.1 (M + 1). Anal. Calcd. For $C_{20}H_{14}N_2O_4S$: C, 63.48; H, 3.73; N, 7.40; S, 8.47. Found: C, 63.42; H, 3.68; N, 7.46; S, 8.41%.

13c: 8-Methyl-2-oxo-2H-chromene-3-carboxylic acid (4-phenyl-thiazol-2-yl)-amide: Yield: 85%. mp 160–162 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3115–3145 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ2.3 (s, 3H, Ar − CH_3), 7.0–7.8 (m, 9H, Ar–H), 8.3 (s, 1H, coumarin ring-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 18.7, 99.9, 114.1, 122.1, 123.8, 125.4, 127.5, 128.6, 128.8, 129.3, 131.7, 133.0, 138.5, 148.1, 150.4, 159.4, 163.0, 164.2. LC–MS *m/z* 363.1 (M + 1). Anal. Calcd. For $C_{20}H_{14}N_2O_3S$: C, 66.28; H, 3.89; N, 7.73; S, 8.85. Found: C, 66.22; H, 3.83; N, 7.77; S, 8.89%.

13d: 8-Methyl-2-oxo-2H-chromene-3-carboxylic acid [4-(4-



Scheme 1. Synthesis of 2-oxo-2H-chromene-3-carboxylic acid *N*'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide analogs (**12a-d**) and 2-oxo-2H-chromene-3-carboxylic acid (4-phenyl-thiazol-2-yl)-amide analogs(**13a-f**). Steps and Reagents: i. K_2CO_3/Acetone, Reflux, ii. $NH_2NH_2.H_2O$/Ethanol, Stirrig, RT, iii. I_2/Ethanol, Reflux, iv. Piperidine/Ethanol, Reflux, v. NaOH/Ethanol, Reflux, vi. TBTU/Lutidine in DCM, stirring, 0–5 °C.

methoxy-phenyl)-thiazol-2-yl]-amide: Yield: 80%. mp 180–182 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3150–3180 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ2.3 (s, 3H, Ar−CH_3), 3.7 (s, 3H, OCH_3), 7.0–7.8 (m, 8H, Ar–H), 8.3 (s, 1H, coumarin ring-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 18.7, 55.8, 99.9, 114.1, 114.8, 122.1, 123.8, 125.4, 128.5, 128.6, 131.7, 138.5, 148.1, 150.4, 159.4, 160.6, 163.0, 164.2. LC–MS *m/z* 393.2 (M + 1). Anal. Calcd. For $C_{21}H_{16}N_2O_4S$: C, 64.27; H, 4.11; N, 7.14; S, 8.17. Found: C, 64.22; H, 4.17; N, 7.19; S, 8.11%.

13e: 7-Methoxy-2-oxo-2H-chromene-3-carboxylic acid (4-phenyl-thiazol-2-yl)-amide: Yield: 81%. mp 220–222 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3115–3145 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 3.7 (s, 3H, OCH_3), 7.0–7.8 (m, 9H, Ar–H), 8.3 (s, 1H, coumarin ring-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 55.8, 99.9, 107.6, 111.0 114.1, 114.5, 114.8, 127.5, 127.8, 128.8, 129.3, 133.0, 138.5, 148.1, 151.1, 159.4, 160.2, 163.0, 164.2. LC–MS *m/z* 379.1 (M + 1). Anal. Calcd. For $C_{20}H_{14}N_2O_4S$: C, 63.48; H, 3.73; N, 7.40; S, 8.47. Found: C, 63.43; H, 3.77; N, 7.46; S, 8.42%.

13f: 7-Methoxy-2-oxo-2H-chromene-3-carboxylic acid [4-(4-methoxy-phenyl)-thiazol-2-yl]-amide: Yield: 89%. mp 184–186 °C. FT-IR (KBr, cm^{-1}): 1630 (C═O), 1670 (C═O, amide), 3135–3160 (N-H). ^{1}H NMR (400 MHz) (DMSO-d_6): δ 3.7 (s, 6H, OCH_3), 7.0–7.8 (m, 8H, Ar–H), 8.3 (s, 1H, coumarin ring-H), 9.5 (s, 1H, NH). ^{13}C NMR (100 MHz) (DMSO-d_6) δ: 55.8, 99.9, 107.6, 111.0 114.1, 114.5, 114.8, 125.3, 127.8, 128.5, 138.5, 148.1, 151.1, 159.4, 160.2, 160.6, 163.0, 164.2. LC–MS *m/z* 409.1 (M + 1). Anal. Calcd. For $C_{21}H_{16}N_2O_5S$: C, 61.75; H, 3.95; N, 6.86; S, 7.85. Found: C, 61.70; H, 3.90; N, 6.81; S, 7.81%.

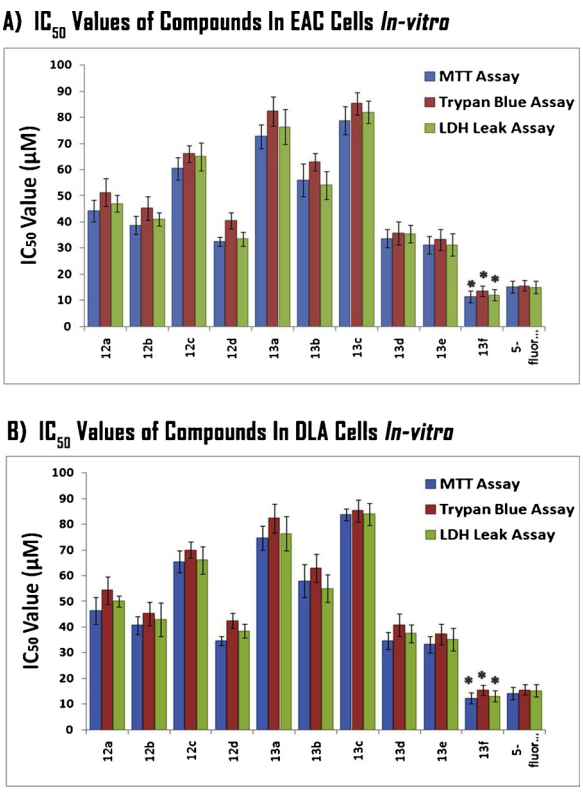


Fig. 1. *In vitro* anti-neoplasmic evaluation (IC_{50} value) of cytotoxic compounds on EAC and DLA cells using three different cytotoxicity assays like MTT, trypan blue and LDH leak assays. Compound **13f** shows the IC_{50} values. **(A)** at around 12.3 µM in EAC tumour cells and **(B)** at around 15.0 µM in DLA tumour cells. (Statistical significant value is *p ≤ 0.05).

Table 1
Evaluation for the side effects of the compound **13f** by subjecting to hematological and serum profile of non tumour bearing mice.

Hematological and serum profile parameters	Normal mice	Treated mice
Alkaline Phosphatase (IU/L)	133.78 ± 1.30	136.45 ± 2.3
Creatinine (mg/dl)	0.39 ± 1.3	0.42 ± 1.15
Urea (mg/dl)	45 ± 2.2	41 ± 2.5
RBC (10^6/µl)	5.5 ± 0.4	5.2 ± 0.8
WBC (10^6/µl)	3.56 ± 2.2	3.6 ± 2.5

Values are indicated in mean ± SEM.

2.2. Biology

2.2.1. Evaluation of in vitro cytotoxic activity of the compounds *12a-d* and *13a-f*

The cytotoxic effect of the synthesized compounds 12a-d and 13a-f was evaluated against Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma Ascites (DLA) cells *in-vitro* using trypan blue dye exclusion, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactic acid dehydrogenase (LDH) leak assay [23] The cells were cultured in Dulbecco's modified eagle medium (DMEM) medium (Gibco-Invitrogen, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, USA), Penicillin-Streptomycin (Sigma-Aldrich, USA) and sodium bicarbonate (0.37%) and incubated at 37 °C in an atmosphere of 95% air and 5% carbon dioxide, with 98% humidity. The cells were treated using increasing concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µM in DMSO) of compounds 12a-d and 13a-f for various time intervals (0–48 h), while the controls received only DMSO. Besides, 5-fluorouracil is used as a standard and each experiment was repeated for a minimum of three independent times.

2.2.2. BrdU incorporation assay

The BrdU incorporation assay was performed for validating compound **13f** effect on EAC proliferation. In brief, about 20,000 EAC cells were seeded on coverslips placed in a 30 mm dish with complete growth media. The cells were treated with and without compound **13f** (10 µM) and after 24 h, the cells were labeled using 10 µM BrdU (Sigma Aldrich, USA) and re-incubated for 48 h. After incubation the culture media was removed and the cells were fixed using 75% ethanol for 10 min. The coverslips were blocked with 1% ovalbumin and probed with anti-mouse BrdU primary antibody (Santacruz, USA) for 1 h at room temperature and then incubated with anti-mouse Alexaflouro 564 secondary antibody followed by washing and mounted on coverslips using antifade gold mounting solution. Finally, the samples were observed under EVOS FL cell imaging, Thermo Scientific, USA.

2.2.3. Determination of LD_{50} and in vivo evaluation for the side effects of the compound *13f*

Swiss albino male mice weighing 25–28 g were housed under standard laboratory conditions in accordance with the CPCSEA guidelines for laboratory animal facility. All procedures used for animal experimentation were approved by the Institutional Animal Ethical Committee, National College of Pharmacy, Shimoga, India, (NCP/IAEC/CL/101/05/2012-13). The compound **13f** was subjected to short term acute toxicity studies in normal, healthy Swiss albino mice divided into 5 groups (n = 6) by injecting intraperitoneally (IP) and LD_{50} was determined as per the standard CPCSEA guidelines.

To evaluate the side effects of compound **13f**, normal Swiss albino mice was injected with compound **13f** (75 mg/kg body weight, i.p) for ten doses continuously for ten days (n = 6). The physiological functions of mice of both treated and untreated groups were evaluated by collecting blood on the 11^{th} day. Serum was separated from the blood and used for liver and kidney function tests by comparing the levels of alkaline phosphatase (ALP), creatinine and urea. The blood count was performed by collecting plasma and the number of RBC and WBC were noted down [28,29].

2.2.4. In-vivo tumour model and treatment with compound *13f*

The *in vivo* antitumour effect of the compound **13f** was verified against Ehrlich ascites tumour. The EAC cell suspensions were prepared with phosphate buffer saline (PBS) and maintained in the peritoneal cavity of mice by injecting 0.2 ml of ascitic fluid containing 5×10^6 cells/mouse for every 10 days. An ascitic tumour model was developed by injecting EAC cells in the peritoneum region of mice intraperitoneally and tumour growth was noticed. The mice bearing EAC were administered with compound **13f** (75 mg/kg body weight i.p) for three doses on every alternate day after the onset of tumour on the 4^{th} day and evaluated for cell count. The ascites volume and survivability analysis were carried out as reported earlier [18].

2.2.5. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay

In this assay, initially the animals with EAC were treated with and without compound **13f**. After 24 h, the cells were harvested and 100 µL of cells were added on 10 mm coverslips and incubated for 1 h for cells to settle. Then the cells were fixed on it using 4% paraformaldehyde for about 20 min and washed with PBS three times with the interval of 5 min. The cells were permeabilized using 0.25% Triton-x 100. TUNEL assay was performed according to the manufacturer's instructions (Thermo Scientific, USA).

2.2.6. Fluorescent activated cell sorting (FACS)

The EAC cells were treated with or without compound **13f** *in vivo* was harvested and stained with propidium iodide for determining the cell death as previously described [30,31].

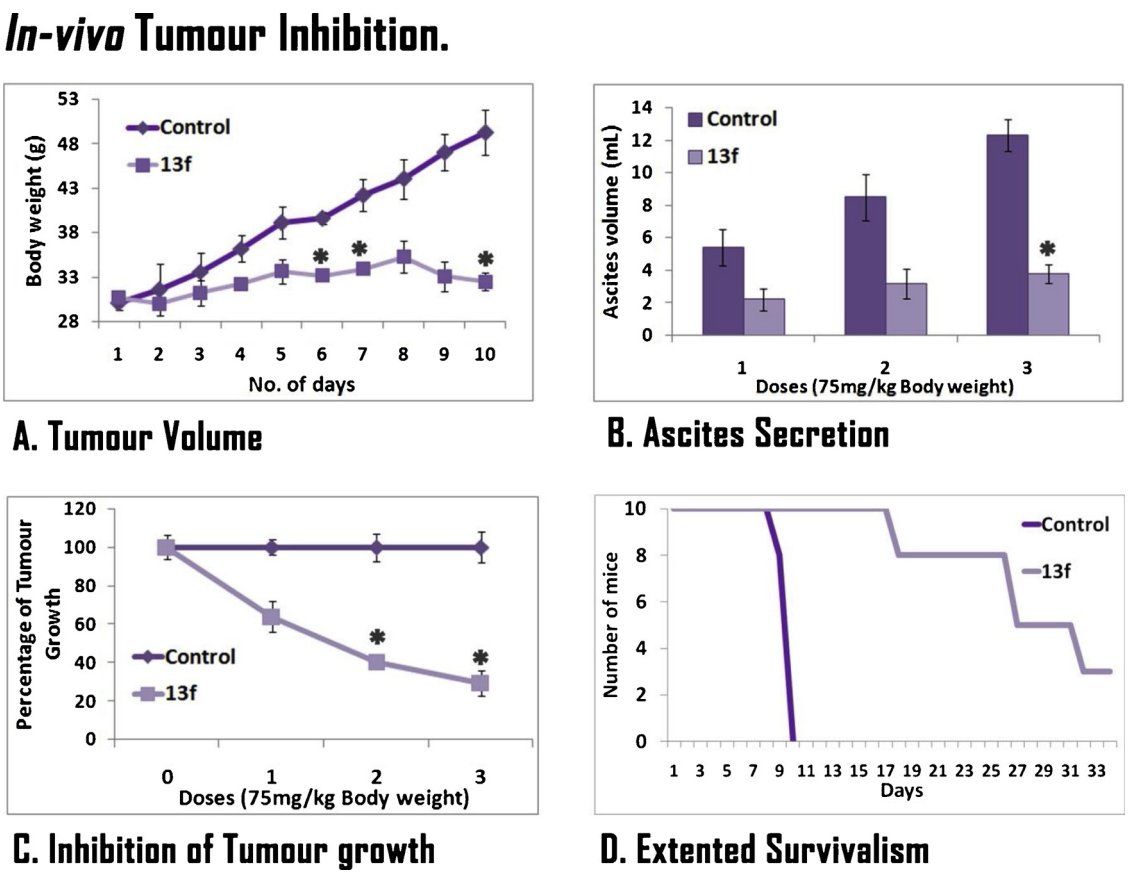


Fig. 2. *In vivo* tumour inhibitory effect of compound **13f** in ascites carcinoma mice. EAC cells were cultured *in vivo* and administered plus or minus compound **13f** (75 mg/kg body weight, i.p). (**A**) Decrease in tumour volume by measuring body weight. (**B**) Compound **13f** decreases the secretion of ascites in a dose dependent manner. (**C**) Dose dependent tumour growth reduction after compound **13f** treatment. (**D**) Cumulative survivality curve for control and compound **13f** treated mice. (Statistical significant value is *p ≤ 0.05).

2.2.7. Western blot

The lysates were prepared by using RIPA buffer [30,31] from *in vivo* with or without compound **13f** treated EAC cells, quantified and after SDS-PAGE, proteins were transferred to PVDF membrane. Western blots were performed for cleaved caspase-8 (Santacruz, USA), cleaved caspase-3 (Santacruz, USA), caspase activated DNase (CAD)(Santacruz, USA), cleaved poly(ADP)ribosylation polymerase (PARP) (Santacruz, USA), H2A histone family member X (γ-H2AX) (Santacruz, USA) and β-actin (BD Bioscience, USA). Relative normalized expressions were calculated by Imagej FiJi software.

2.2.8. DNA fragmentation assay

The *in vivo* with or without compound **13f** treated EAC cells were harvested for the isolation of genomic DNA as described earlier [30–32]. The isolated DNA was electrophoresed on 1.5% agarose gel at 50 V using Tris/Borate/EDTA (TBE) buffer and documented using Bio-rad gel documentation ™XR + Imaging System.

2.2.9. Molecular docking studies

Docking was performed on windows 2007 using MOE 2008.10 version. Human caspase 3 was imported from the protein data bank (PDB ID: 4QTX) and the enzyme was visualized using sequence option and non interacting water molecules were removed. The partial charge of protein was adjusted, using the force field method AMBER 99. Later, the protein was subjected to 3D protonation at cut off 12.0, and further hydrogen was added according to standard geometry and the enzyme was energy minimized using force field MMFF94x at 0.01 KJ per mole gradients. The ligand preparation was done by drawing the structure of ligand by using a builder module, and adjusting the partial charges using Hamilton MMFF94 force field method and subsequently 3D protonation and hydrogen addition was performed according to standard geometry. Ligands were energy minimized at cut off 12 using MMFF94x force field at 0.01KJ per mole gradient. Docking was performed using the option simulation followed by dock on selected active site amino acids using sequence option, and further docked with setting options such as: receptor and solvent, selected residues, alpha triangle, affinity dG, force field refinement and best 10 pose. After obtaining docking results, out of the 10 best posed resulted for each chemical structure, best pose was retained. The resultant best pose score values in the series were used for analysis of docking and interaction.

3. Results and discussions

3.1. Chemistry

The title compounds **12a-d** and **13a-f** were synthesized by multi step synthetic procedure as shown in Scheme 1. All the synthesized compounds were established by IR, ^{1}H NMR, ^{13}C NMR and mass spectral data, along with few single X-ray crystallographic data [23]. Compound **3** [ethyl (quinolin-8-yloxy)-acetate] [20], which is obtained by the condensation of 8-hydroxy quinoline (**1**) and ethyl chloro acetate (**2**) was confirmed from NMR spectrum by the disappearance of broad singlet of compound **1** for OH proton and the appearance of triplet and quartet for the CH_3 and CH_2 proton peaks respectively, confirms the formation of product **3** [23]. Further, compound **3** reacted with hydrazine hydrate to give quinoline acetic hydrazide **4** [(quinolin-8-yloxy)-acetic hydrazide], which was confirmed by the disappearance of carbonyl stretching of compound **3** and the appearance of the N—H

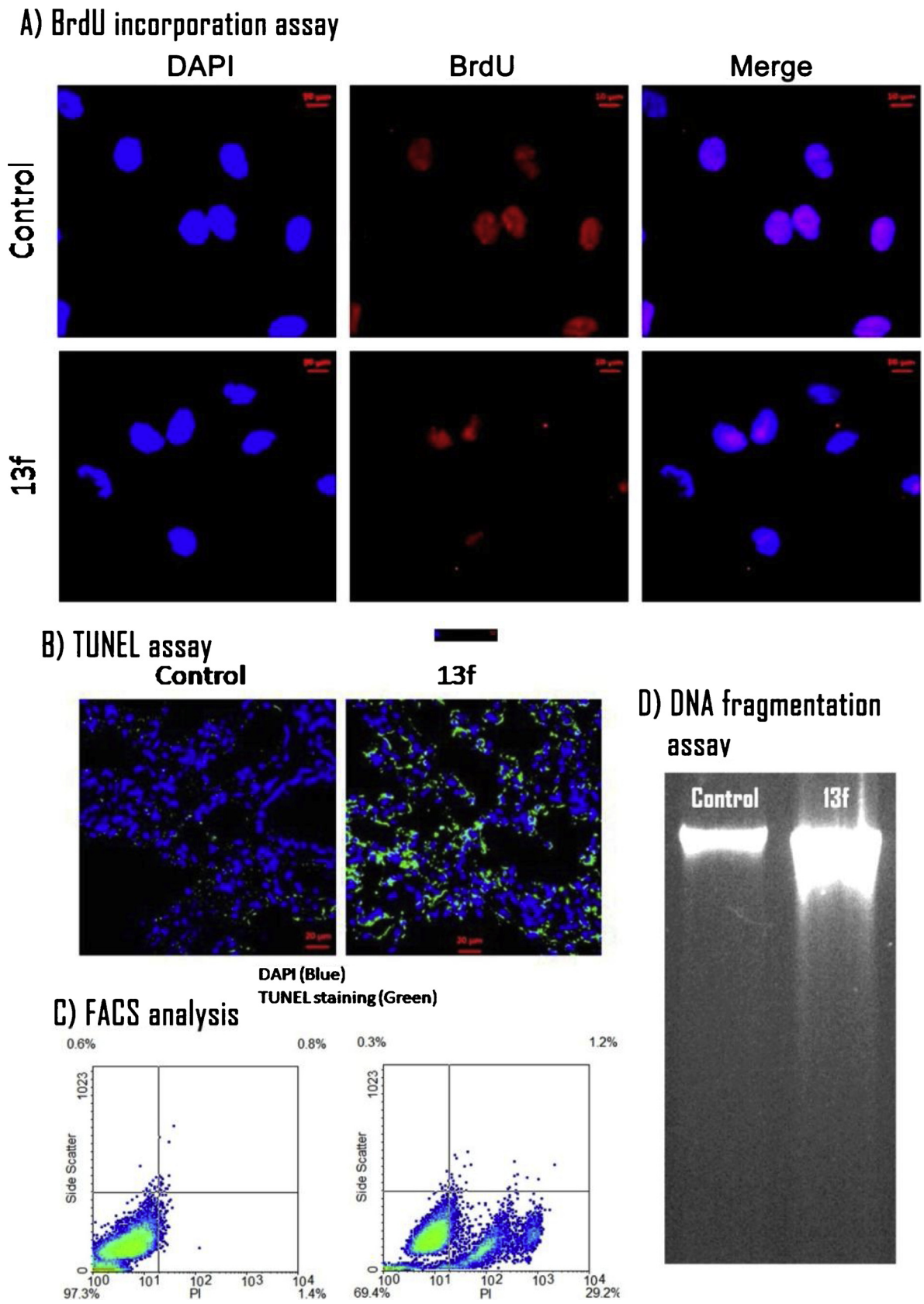


Fig. 3. Compound **13f** induces programmed cell death against EAC proliferation. (**A**) Compound **13f** exhibited inhibition of EAC cell proliferation *in vitro* as verified by BrdU incorporation assay (red colour stain indicates the rate of DNA replication). (**B**) Compound **13f** exposed the apoptotic effect in ascitic carcinoma *in vivo* as studied by TUNEL assay (Green colour indicates DNA breaks in EAC cells). (**C**) Compound **13f** induced the apoptogenesis (29.2%) in EAC cells as validated by FACS analysis. (**D**) Compound **13f** treatment induced the DNA fragmentation in EAC cells.

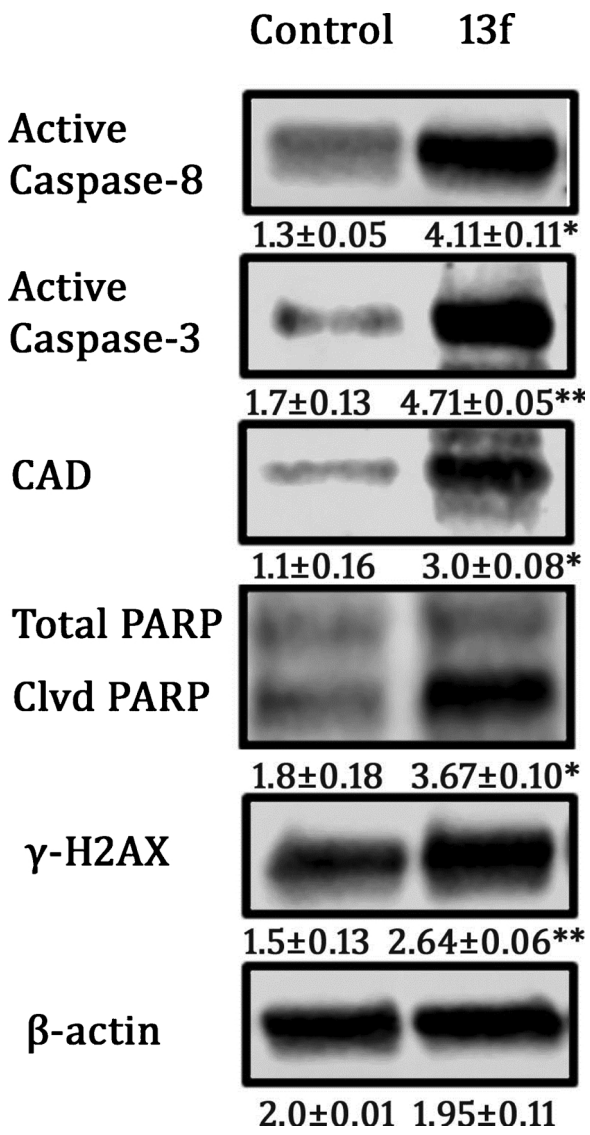


Fig. 4. Compound **13f** induces critical apoptotic/DNA fragmentation factors *in vivo*. Western blots performed in three independent times for cleaved caspase-8, cleaved caspase-3, CAD, cleaved PARP and γ-H2AX. Further, imajeJ software was used to calculate the relative expression of the genes. Blots panel shows the altered expressions of critical apoptotic/DNA fragmentation factors. Values were expressed as mean ± standard error and statistically significant values were depicted as *p ≤ 0.05 and **p ≤ 0.01.

stretching band in the IR spectrum and also by the appearance of broad singlet for N—H proton and the disappearance of CH_3 and CH_2 proton peaks of compound **3** in proton NMR [24]. 2-Amino-4-phenyl thiazoles (**7a-b**) were obtained by treating substituted acetophenones and thiourea in the presence of iodine as a catalyst, which were recognized an increase in one aromatic proton of thiazole ring and the disappearance of one NH_2 proton from thiourea in the NMR spectra [25]. Compounds **10a-d** were synthesized by knoevenagel condensation and confirmed by the appearance of CH_3 and CH_2 proton peaks along with the appearance of coumarin aromatic proton in the range 9.1–9.3 ppm in NMR spectra [24]. Further, compounds **10a-d** on hydrolysis afforded coumarin-3-carboxylic acids (**11a-d)** which were confirmed by the disappearance of CH_3 and CH_2 proton peaks of compounds **10a-d** and as well as by the appearance of carboxylic acid proton of compounds **11a-d** in the range 13.0–13.3 ppm in NMR spectra [27]. Finally, compounds **12a-d** [2-oxo-2H-chromene-3-carboxylic acid *N*'-[2-(quinolin-8-yloxy)-acetyl]-hydrazide analogs] and **13a-f** [2-oxo-2H-chromene-3-carboxylic acid (4-phenyl-thiazol-2-yl)-amide analogs] were synthesized in good yield by coupling coumarin-3-carboxylic acids **(11a-d)** with quinoline acetic hydrazide (**4)** and 2-amino-4-phenyl thiazoles (**7a-b)** respectively, by using TBTU (O-(benzotriazol-1-yl)- N,N,N',N'-tetramethyl uranium tetrafluoroborate) as a coupling agent. This was supported by the disappearance of NH_2 and COOH stretching of compounds **4** and **11a-d** respectively, in the IR spectra. Also, by the disappearance of the broad peak of NH_2 and COOH protons and appearance of CONH proton of compounds **12a-d** and **13a-f** in the NMR spectra.

3.2. Biology

3.2.1. Compound *13f* exhibits strong cytotoxicity and emerged as a lead compound

In our ongoing efforts to discover the chemotherapeutic anti-cancer agents, the synthesized quinoline **12a-d** and thiazole **13a-f** analogs containing coumarin moiety were initially tested for their cytotoxic and antiproliferative activity in the EAC and DLA cells *in vitro*. The compounds were verified for their activity using MTT, trypan blue and LDH leak assay. These methods were opted to select the active and potent compounds within the series. In the synthesized series, the compound **13f** with a methoxy group at the para position in the phenyl ring attached to thiazole, along with another methoxy group at seventh position in coumarin ring has shown effective antiproliferation against EAC cells with IC_{50} values of 11.4 μM, 13.5 μM and 12.1 μM in MTT, trypan blue and LDH leak assay respectively (Fig. 1(A)). Comparable results were obtained against DLA cells with IC_{50} values of 15.3 μM, 15.6 μM and 14.2 μM in MTT, trypan blue and LDH leak assay respectively (Fig. 1(B)). Among the synthesized analogs, compound **13f** exhibited more cytotoxicity and antiproliferative effect than other analogs and unquestionably emerged as most active and lead compound within the series.

3.2.2. Structure activity relationship (SAR)

The literature survey revealed that the nature and positions of different substituents in a compound are responsible for the target biological activity. Also the analysis of SAR facilitates to modify the effect or the potency of the compound by changing its chemical structure. Besides, the pharmacological, biochemical properties and therapeutic applications of coumarins depends on the pattern of substitution and have attracted intense interest in recent years because of their diverse pharmacological properties [33]. In support of this, the present investigation involves the multistep synthesis of coumarin analogs conjugated with quinoline **12a-d** and thiazole **13a-f** moieties. Structurally, the compounds are having a basic backbone of coumarins with quinoline and thiazole with a methoxy group at the para position in the phenyl ring, along with halogen, methyl and methoxy groups at different positions in the coumarin moiety. The IC_{50} values as displayed in Table 1, suggest that the compound **13f** with one electron donating methoxy group at the seventh position of the coumarin ring and another electron donating methoxy group at the para position in the phenyl ring of thiazole shown IC_{50} values as 15.3 μM, 15.6 μM and 14.2 μM in MTT, trypan blue and LDH leak respectively assay (Fig. 1(B)). Subsequently, compound **13f** was selected as a lead compound for further evaluation, based on its significant structure activity relationship compared with the other analogs in the series. The replacement of a hydrogen atom of inactive compound **13a** by bulky electron donating methoxy substituents as in compound **13f** had a positive effect on the apoptogenic activity.

3.2.3. Compound 13f inhibits tumour growth In-vivo

In vivo murine ascites tumour is the reliable model system to study the preliminary antitumour potency of a cytotoxic molecule. It is a rational and a hierarchical approach beginning with toxicology and pharmacology studies, progressing to primary tumours to identify

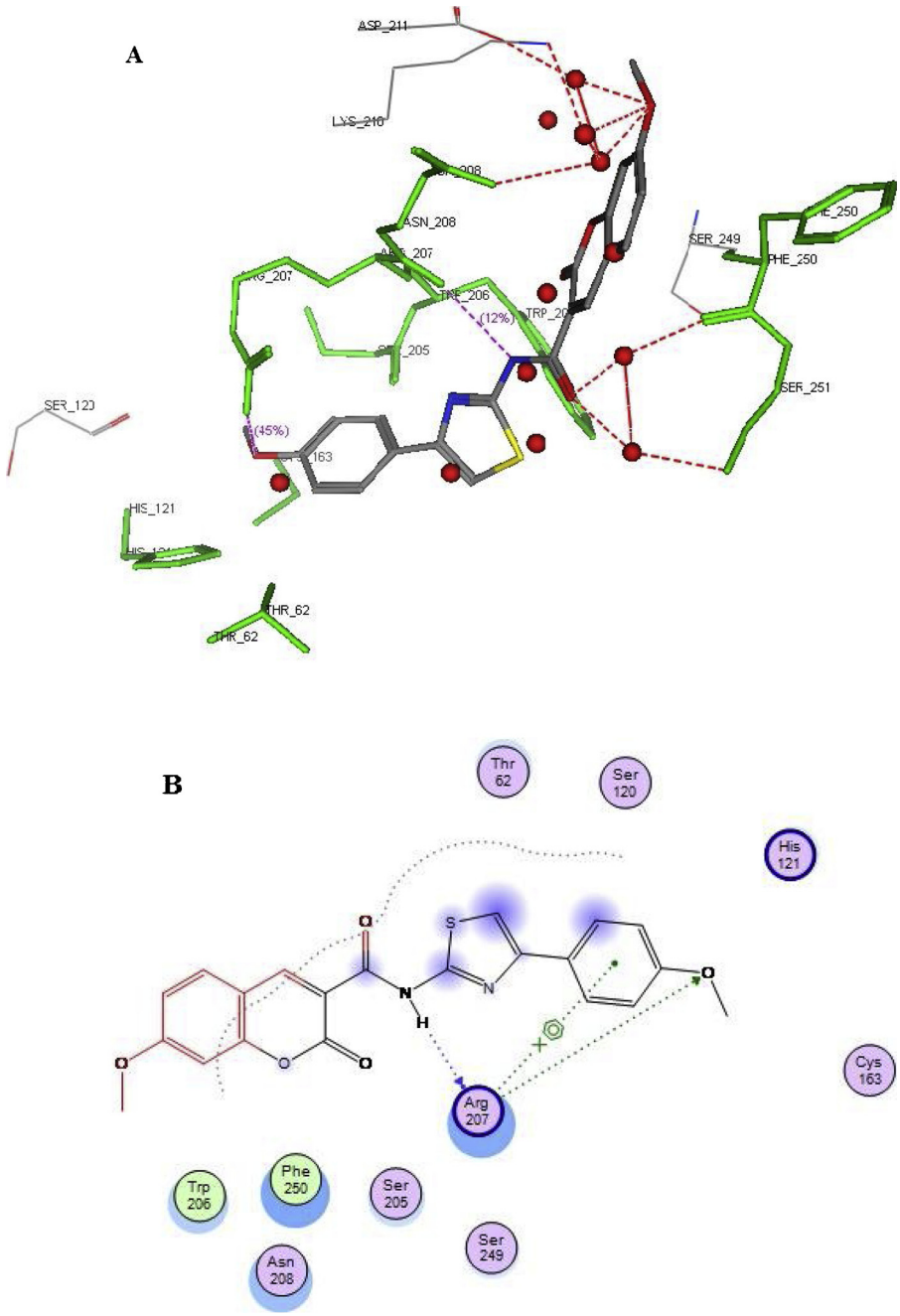


Fig. 5. (**A**) 3D structure of compound 13**f** interacting with human caspase 3. (**B**) H-bond interaction of the ligand compound 13**f** interacting with human caspase 3.

therapeutic targets and models of metastatic disease to compare drugs using rigorous, clinically relevant outcome parameters [18]. In the present study, the lead compound **13f** was induced in EAC bearing mice at 75 mg/kg body weight intraperitoneally, on every alternative day after the onset of tumour for three doses. Upon treatment with the compound **13f**, we observed dose dependent decrease of the body weight (Fig. 2(A)). In correlating to this, the ascites secretion in compound **13f** treated animals was also reduced when compared to untreated control (Fig. 2(B)). It is well known from several studies that the decrease in ascites fluids accounts for the suppression of tumour growth [35]. In relation, we observed dose dependent reduction in tumour growth of ~76% after treatment with the compound **13f** (Fig. 2(C)) eventually leading to the expanded life span of animals from the 11th day to 34th day showing three fold increases in survivalism (Fig. 2(D)). On the other hand, the compound **13f** was assessed for the toxicological effect on non tumour bearing animals by assessing the liver function test and blood count. The treatment with compound **13f** showed less or no toxicological side effects and the results are summarized in Table 1.

3.2.4. *Compound 13f induces apoptosis in EAC cells*

Recent evidence supports the concept that tumour growth in-vivo depends on evasion of normal homeostatic control mechanisms that operate through induction of cell death by apoptosis. Reduction in tumour growth may be because of abridged proliferation and then leading to programmed cell death of the regressing tumour [2]. Our results suggest that there was a drastic decrease in tumour growth after the treatment with compound **13f**. In this context, we further verified the effect of compound **13f** on the induction of programmed cell death in EAC cells. The BrdU incorporation in the cells of control and compound **13f** treated revealed that the EAC cells lost their proliferating effect upon treatment with compound **13f** under in-vitro (Fig. 3(A)). The loss of proliferating ability of the EAC cells may lead the cells to undergo

programmed cell death. Further, we performed TUNEL staining to assess the compound **13f** induced apoptosis in EAC cell proliferation. Perceptibly, the TUNEL staining shown apoptotic EACs in the compound **13f** treated under in-vivo (Fig. 3(B)). Moreover, FACS results also indicate that the compound **13f** have increased the cell population undergoing apoptosis about 29.2% compared to untreated, which firmly ascertains the potential of the compound **13f** in promoting cell death under in-vivo (Fig. 3(C)). On verifying the possible genotoxic effect of compound **13f** on EAC cells in-vivo by DNA degradation assay, we found that the DNA fragmentation efficacy of compound **13f** was also apparent by DNA degradation in treated and confirms that **13f** specifically targets programmed cell death in ascites tumour (Fig. 3(D)). With this convincing results of functional assay system, we extended the study to validate the apoptotic/DNA damaging critical factors underlying compound **13f** mediated tumour regression. The cleavage of caspase-8, caspase-3 and PARP plays essential role in the activation of CAD mediated DNA damage and γ-H2AX accumulated the site of DNA damage and acts as a biomarker to measure the DNA breaks[32,36]. Western blot results postulated that **13f** induced various crucial apoptotic/DNA fragementation factors such as cleaved caspase-8, cleaved caspase-3, CAD, cleaved PARP and γ-H2AX (Fig. 4), which leads to **13f** induced tumor cell death.

*3.2.5. Compound **13f** interacts strongly with caspase 3 in docking studies*

The molecular docking was performed as per the standard procedure [37] and the analysis revealed that, the synthesized coumarin appended with thiazole and quinoline amides (**12a-d and 13a-f**), interacted with active sites of amino acids in caspase 3 from human source. Except **12b**, the other compounds in the series interacted with either Arg 207 or Asn 208, *via* hydrogen acceptor or hydrogen donor type of bond. Further, for some molecules even caspase 3 active site water is also found to interact with above mentioned amino acids, all the molecules showed single bond interaction, except 12b (no interaction). Compound **13f** exhibited three interactions with Arg 207 involving hydrogen donor bond with amide hydrogen of the **13f** and Arg 207. The second interaction is hydrophobic which involves nitrogen atom of active site of the target caspase 3 with phenyl ring of **13f** and the last interaction is hydrogen acceptor type of bond between Arg 207 and oxygen atom of methoxy group attached to phenyl ring of **13f**. This molecular docking study was well correlated with western blot caspase 3 assay (Fig. 5).

4. Conclusions

A series of novel coumarin analogs **12a-d** and **13a-f** were synthesized. In this series, the compound **13f** with a methoxy group at the para position on the phenyl ring of thiazole, along with another methoxy group at seventh position in coumarin ring has shown a promising antiproliferative effect against two different cell lines of different origin in an MTT, tryphan blue and LDH leak assay with 5-fluorouracil as a standard and emerged as the lead compound within the series. Further, the anti-neoplastic effect of the compound **13f** was verified against Ehrlich ascites tumour by BrdU incorporation, TUNEL, FACS and DNA fragmentation assay and also by docking studies. Experimental data shown that compound **13f** induces apoptotic cell death by degrading genomic DNA of cancer cells through cleaved caspase-8, cleaved caspase-3, CAD, cleaved PARP and γ-H2AX and thereby decreasing the ascitic tumour development in mice.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.108707.

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