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Synthesis and molecular docking of novel non-cytotoxic anti-angiogenic sulfonyl coumarin derivatives against hepatocellular carcinoma cells *in vitro*

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ABSTRACT

Resistance to conventional cytotoxic therapeutics, emphasize the need for efforts to develop non-cytotoxic targeted molecular therapies directed against the pathways involved in the angiogenesis. In this work a new series of coumarin derivatives was synthesized starting from 2-oxo-2*H*-coumarin-6-sulfonyl chloride (**1**), 6-nitro-2-oxo-2*H*-coumarin-3-sulfonyl chloride (**10**) and 6-amino coumarin-2-one (**19**). The tested compounds **4**, **5**, **8**, **12**, **13** and **14** were non-cytotoxic against hepatocellular carcinoma cells (HepG2) using MMT. These non-cytotoxic compounds were evaluated as anti-angiogenic agent. Results revealed that compounds **8** and **12** exhibited MMP-independent anti-migratory activity, while compounds **4**, **5**, **8**, **13** and **14** induced MMP-dependent anti-migratory activity against hepatocellular carcinoma. Therefore, these coumarin molecules can be utilized as lead compounds to develop potential non-toxic angiogenesis inhibitors and small molecular ligands to target (HepG2). Compound **4** considered a promising anti-angiogenic agent, where it exhibited MMP-dependent anti-migratory activity and down regulated CD105. Furthermore, the molecular docking of the tested compounds was carried out in order to investigate their binding pattern with the prospective target, MMP-2 (PDB-code: 1HOV). The docking results indicate that all tested compounds exhibited better docking score and good fitting inside the active side of MMP-2 (PDB-code: 1HOV) which was in concomitant with biological results.

INTRODUCTION

Angiogenesis is a normal process, required for normal tissue repair and growth (Ucuzian*et al*., 2010). Angiogenesis is the key factor in the development and metastasis of a variety of tumor types, and represent an important hallmark of malignant disease (Ma and Waxman, 2008). The inhibition of tumor growth by anti-angiogenic drugs has been achieved both in preclinical studies and in clinical trials, where promising anti-tumor responses have been reported for a variety of anti-angiogenic agents (eg. bevacizumab, sunitinib, and sorafenib) (Vasudev and Reynolds 2014). Overall, the survival benefits of anti-angiogenic drugs leading to increased interest in developing more effective ways to combine antiangiogenic drugs with traditional cytotoxic chemotherapies (Folkman 2007; Cesca 2013). Over the last years, several non-cytotoxic molecular targeted therapies have been developed against growth factor receptors and tumor angiogenesis (Idbaih *et al*., 2008). Coumarins constitute a class of compounds belong to the family of lactone, which are found widely in nature (Keating and O'Kennedy 1997), and possess diverse biological activities (Al-Bayati *et al*., 2010), for example, antimicrobial (Sahoo *et al*., 2015), anti-inflammatory (Kirsch *et al*., 2016), antioxidant (Arora *et al*., 2014) and antiviral including human immunodeficiency virus (HIV) (Curini *et al*., 2003; Završnik *et al*., 2011). In cancer drug development arena, coumarin-type compounds have been reported to bosses marked cytotoxic activities (Kostova *et al*., 2005; Jeon *et al*., 2015), in addition act as novel angiogenesis inhibitors. From this perspective, the present work is aimed to illustrate the anticancer activity of novel sulfonyl coumarin derivatives.

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In addition we attempted to explore the probability of the most promising anti-angiogenic compounds to inhibit matrix mettaloproteinase enzyme *via* molecular docking study of these compounds against the active site of the protein molecular surface of MMP-2 (PDB ID: 1HOV)

MATERIALS AND METHODS

Instruments and reagents

Melting points were determined on the digital melting point apparatus (Electro thermal 9100, Electro thermal Engineering Ltd, serial No. 8694, Rochford, United Kingdom) and are uncorrected. The micro analytical data were achieved on a Perkin-Elmer 2400 analyzer (Perkin-Elmer, 940 Winter Street, Waltham, Massachusetts 02451, USA) and were found within ±0.4 % of the theoretical values. IR spectra were recorded on a Perkin-Elmer 1600 Fourier Transform Infrared Spectrophotometer using KBr discs. The ^{1}H NMR spectra were measured with a Bruker Avance digital spectrometer (BRUKER BioSpin GMBH Silberstreifen D-76287 Rheinstetten, Germany) (500 MHz) in DMSO-*d*6, and chemical shifts were recorded in δ ppm relative to TMS as internal standard (all NH_2 and NH recorded for the compounds were D_2O-exchangeable).Mass spectra (EI) were recorded at 70 eV with JEOL-JMS-AX500 mass spectrometer (JEOL Ltd. 1-2, Musashino 3-chome Akishima, Tokyo 196-8558, Japan). All reagents and solvents were of commercial grade. 2-oxo-2*H*-coumarin-6-sulfonyl chloride (**1**) (Ismail *et al.*, 1989), 6-nitro-2-oxo-2*H*-coumarin-3-sulfonyl chloride (**10**) (Abd El-Hafez *et al.*, 1994), 2-cyanoacetic acid hydrazide (Heibron 1965), 2'-acetyl-2-cyanoacetohydrazide (Graham *et al.*, 1949), 3-amino-5-pyrazolone (Callejo *et al.*, 1990), and arylidene malononitriles (Kassem *et al.*, 2012) were prepared as reported.

Synthesis

N'-(2-Cyanoacetyl)-2-oxo-2H-chromene-6-sulfonohydrazide (2)

A mixture of 2-oxo-2*H*-chromene-6-sulfonyl chloride (**1**) (2.4 g, 0.01 mol) and 2-cyanoacetic acid hydrazide (1.3 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was stirred for 3 h at room temperature. The formed precipitate was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 62%; MP:157-9 °C; IR (KBr, ν_{max}, cm^{-1}) 3230, 3165 (NH), 2205 (CN), 1705, 1654 (C=O), 1525 (C=C), 1385, 1117 (SO_2-N), 1135, 1010 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.52 (1H, s, NH), 8.28 (1H, s, H-5), 8.07 (1H, d, H-7), 7.81 (1H, d, H-4), 7.44 (1H, d, H-8), 7.15 (1H, s, NH), 6.32 (1H, d, H-3) 4.15 ppm (2H, s, CH_2); MS (*m/z*): 307 [M$^+$]; Anal. $C_{12}H_9N_3O_5S$ (307.28): Calcd: C, 46.90; H, 2.95; N, 13.67; Found: C, 46.81; H, 2.84; N, 13.52.

5-Amino-1-(2-oxo-2H-chromene-6-sulfonyl)-1H-pyrazol-3(2H)-one (3)

A solution of compound **2** (2.4 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was heated under reflux for 3 h. After cooling, the solid that formed was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 65%; MP: 184 dec °C; IR (KBr, ν_{max}, cm^{-1}) 3410, 3325 (NH_2), 3265 (NH), 1710, 1696 (C=O), 1556 (C=C), 1375, 1126 (SO_2-N), 1133, 1019 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.09 (1H, s, NH), 8.30 (1H, s, H-5), 8.12 (1H, d, H-7), 7.81 (1H, d, H-4), 7.45 (1H, d, H-8), 6.41 (1H, d, H-3), 4.54 (1H, s, pyrazolyl H-4), 2.16 ppm (2H, s, NH_2); MS (*m/z*): 307 [M$^+$]; Anal. $C_{12}H_9N_3O_5S$ (307.28): Calcd: C, 46.90; H, 2.95; N, 13.67; Found: C, 46.82; H, 2.81; N, 13.55.

1-Acetyl-5-amino- 4-(2-oxo-2H-chromene-6-sulfonyl)-1,2-dihydro-pyrazol-3-one (4)

A mixture of **1** (2.4 g, 0.01 mol) and 2'-acetyl-2-cyanoacetohydrazide (0.14 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was stirred for 3 h at room temperature, the formed precipitate was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 85%; MP: 102-4 °C; IR (KBr, ν_{max}, cm^{-1}) 3426, 3346 (NH_2), 3165 (NH), 1705, 1686, 1665 (C=O), 1523 (C=C), 1365, 1156 (SO_2), 1142, 1026 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.55 (1H, s, NH), 8.40 (1H, s, H-5), 8.23 (1H, d, H-7), 7.79 (1H, d, H-4), 7.52 (1H, d, H-8), 6.51 (1H, d, H-3), 4.25 (2H, s, NH_2), 1.62 ppm (3H, s, CH_3); MS (*m/z*): 349 [M$^+$]; Anal. $C_{14}H_{11}N_3O_6S$ (349.32): Calcd: C, 48.14; H, 3.17; N, 12.03; Found: C, 48.03; H, 3.01; N, 11.90.

2-Oxo-2H-chromene-6-sulfonic acid (5-oxo-4,5-dihydro-1H-pyrazol-3-yl)amide (5)

A mixture of **1** (2.4 g, 0.01 mol) and 3-amino-5-pyrazolone (0.99 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was stirred for 3 h at room temperature, the formed precipitate was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 77%; MP: 254-6 °C; IR (KBr, ν_{max}, cm^{-1}) 3326 (NH), 1705, 1688 (C=O), 1618 (C=N), 1565 (C=C), 1376, 1145 (SO_2-N), 1133, 1106 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.81 (1H, s, NH), 8.28 (1H, s, H-5), 8.01 (1H, d, H-7), 7.66 (1H, d, H-4), 7.37 (1H, d, H-8), 6.66 (1H, d, H-3), 4.54 (2H, s, CH_2), 2.41 ppm (1H, s, NH); MS (*m/z*): 307 [M$^+$]; Anal. $C_{12}H_9N_3O_5S$ (307.28): Calcd: C, 46.90; H, 2.95; N, 13.67; Found: C, 46.79; H, 3.11; N, 13.53.

Synthesis of pyrano(2,3-c)pyrazoles 6a-d

A solution of compound **5** (3.06 g, 0.01 mol) and an appropriate arylidene malononitriles (0.01 mol) in dry 1,4-dioxane containing triethylamine (1 ml) was refluxed for 4-6 h. After cooling, the solid that formed was filtered off, washed with water, air dried and crystallized from dry ethanol.

2-Oxo-2H-chromene-6-sulfonic acid (6-amino-5-cyano-4-phenylpyrano(2,3-c)pyrazol-3-yl)amide (6a)

Yield: 55%; MP: 221-3 °C; IR (KBr, ν_{max}, cm^{-1}) 3366 (NH_2), 3153 (NH), 2207 (CN), 1710 (C=O), 1618 (C=N), 1545 (C=C), 1385, 1128 (SO_2-N), 1117, 1022 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.85 (1H, s, NH), 9.35 (2H, s, NH_2), 8.53-7.06 (9H, m, Ar-H), 6.82 ppm (1H, d, H-3); MS (*m/z*): 459 [M$^+$];

Anal. $C_{22}H_{13}N_5O_5S$ (459.43): Calcd: C, 57.51; H, 2.85; N, 15.24; Found: C, 57.39; H, 2.73; N, 15.09.

2-Oxo-2H-chromene-6-sulfonic acid (6-amino-4-(4-chlorophenyl)-5-cyano-pyrano(2,3-c)pyrazol-3-yl) amide (6b)

Yield: 56%; MP: 123-5 °C; IR (KBr, ν_{max}, cm^{-1}) 3422 (NH_2), 3175 (NH), 2210 (CN), 1725 (C=O), 1620 (C=N), 1555 (C=C), 1366, 1132 (SO_2-N), 1120, 1052 (C-O-C), 725 (C-Cl); MS (*m/z*): 493/495 [M^+/ M^++2]; Anal. $C_{22}H_{12}ClN_5O_5S$ (493.88): Calcd: C, 53.50; H, 2.45; N, 14.18; Found: C, 53.61; H, 2.33; N, 14.04.

2-Oxo-2H-chromene-6-sulfonic acid (6-amino-4-(4-hydroxyphenyl)-5-cyano-pyrano(2,3-c)pyrazol-3-yl) amide (6c)

Yield: 60%; MP: 236 dec.°C; IR (KBr, ν_{max}, cm^{-1}) 3445 (OH), 3399 (NH_2), 3169 (NH), 2212 (CN), 1705 (C=O), 1620 (C=N), 1582 (C=C), 1362, 1128 (SO_2-N), 1117, 1109 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.05 (1H, s, OH), 8.95 (1H, s, NH), 8.62-7.10 (8H, m, Ar-H), 6.72 (1H, d, H-3), 2.16 ppm (2H, s, NH_2); MS (*m/z*): 475 [M^+]; Anal. $C_{22}H_{13}N_5O_6S$ (475.43): Calcd: C, 55.58; H, 2.76; N, 14.73; Found: C, 55.46; H, 2.80; N, 14.83.

2-Oxo-2H-chromene-6-sulfonic acid (6-amino-4-(2-nitrophenyl)-5-cyano-pyrano(2,3-c)pyrazol-3-yl) amide (6d):

Yield: 63%; MP: 142-4 °C; IR (KBr, ν_{max}, cm^{-1}) 3386, 3295 (NH_2), 3212 (NH), 2207 (CN), 1710 (C=O), 1618 (C=N), 1552 (C=C), 1368, 1135 (SO_2-N), 1126, 1105 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.15 (1H, s, NH), 8.66-7.15 (8H, m, Ar-H), 6.53 (1H, d, H-3), 1.69 ppm (2H, s, NH_2); MS (*m/z*): 504 [M^+]; Anal. $C_{22}H_{12}N_6O_7S$ (504.43): Calcd: C, 52.38; H, 2.40; N, 16.66; Found: C, 52.21; H, 2.52; N, 16.56.

2-(2-Oxo-2H-chromene-6-sulfonyl)malononitrile (7)

A mixture of **1** (2.4 g, 0.01 mol) and malononitrile (0.6 g, 0.01 mol) in dry ethanol (20 ml) containing triethylamine (1 ml) was refluxed for 3 h. The solid that formed was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 76%; MP: 121-3 °C; IR (KBr, ν_{max}, cm^{-1}) 2195 (CN), 1737 (C=O), 1620 (C=C), 1366, 1171 (SO_2), 1111 (C-O-C); MS (*m/z*): 274 [M^+]; Anal. $C_{12}H_6N_2O_4S$ (274.25): Calcd: C, 52.55; H, 2.21; N, 10.21; Found: C, 52.62; H, 2.35; N, 10.14.

6-(3,5-Diamino-4H-pyrazole-4-sulfonyl)chromen-2-one (8)

To a solution of compound **7** (0.01 mol) in dry ethanol (10 ml) containing few drops of triethylamine, hydrazine hydrate 99% (1 ml, 0.02 mol) was added, and then stirred for 5 h. After cooling, the reaction mixture was poured onto ice-water (50 ml). The solid that formed was filtered off, air dried and crystallized from dry ethanol. Yield: 82%; MP: 177 dec. °C; IR (KBr, ν_{max}, cm^{-1}) 3426 (NH_2), 1712 (C=O), 1620 (C=N), 1562 (C=C), 1175 (SO_2-C), 1122, 1009 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.55 (1H, s, H-5), 8.37 (1H, d, H-7), 7.64 (1H, d, H-4), 7.24 (1H, d, H-8), 6.46 (1H, d, H-3), 5.52 (2H, s, NH_2), 3.72 (1H, s, CH-pyrazole), 2.95 ppm (2H, s, NH_2); MS (*m/z*): 306 [M^+]; Anal. $C_{12}H_{10}N_4O_4S$ (306.30): Calcd: C, 47.06; H, 3.29; N, 18.29; Found: C, 47.12; H, 3.20; N, 18.35.

Synthesis of compounds 9a-c

A mixture of compound **7** (0.01 mol) and urea, thiourea or guanidine hydrochloride (0.01 mol) in dry ethanol (10 ml) containing triethylamine (0.5 ml) was refluxed for 8-10 h. After cooling, the reaction mixture was poured onto ice-water (50 ml) and the solid that formed was filtered off, air dried and crystallized from dry ethanol.

4,6-Diamino -5-(2-oxo-2H-chromene-6-sulfonyl)-5H-pyrimidin-2-one (9a)

Yield: 83%; MP: 173-5 °C; IR (KBr, ν_{max}, cm^{-1}) 3412, 3395 (NH_2), 1710, 1692 (C=O), 1622 (C=N), 1575 (C=C), 1345, 1175 (SO_2-C), 1124, 1035 (C-O-C); MS (*m/z*): 334 [M^+]; Anal. $C_{13}H_{10}N_4O_5S$ (334.31): Calcd: C, 46.71; H, 3.02; N, 16.76; Found: C, 46.63; H, 2.92; N, 16.60.

4,6-Diamino- 5-(2-thioxo-2*H*-chromene-6-sulfonyl)-5*H*-pyrimidin-2-one (9b)

Yield: 79%; MP: 210-2 °C; IR (KBr, ν_{max}, cm^{-1}) 3425 (br. NH_2), 1705 (C=O), 1620 (C=N), 1545 (C=C), 1245 (C=S), 1355, 1135 (SO_2-C), 1133, 1101 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.93 (1H, s, H-5), 8.41 (1H, d, H-7), 8.24 (1H, d, H-4), 7.59 (1H, d, H-8), 6.89 (1H, d, H-3), 5.57 (1H, s, CH-pyrimidine), 3.04 (2H, s, NH_2), 2.31 ppm (2H, s, NH_2); Anal. $C_{13}H_{10}N_4O_4S_2$ (350.37): Calcd: C, 44.56; H, 2.88; N, 15.99; Found: C, 44.43; H, 2.75; N, 15.83.

6-(4,6- Diamino-2-imino-2,5-dihydropyrimidine-5-sulfonyl)chromen-2-one (9c)

Yield: 75%; MP: 225-7 °C; IR (KBr, ν_{max}, cm^{-1}) 3415 (br. NH_2), 1705 (C=O), 1620 (C=N), 1563 (C=C), 1366, 1144 (SO_2-C), 1132, 1017 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.54 (1H, s, H-5), 8.41 (1H, d, H-7), 8.21 (1H, d, H-4), 7.59 (1H, d, H-8), 6.66 (1H, d, H-3), 4.11 (4H, s, $2NH_2$), 3.04 ppm (2H, s, NH_2); MS (*m/z*): 333 [M^+]; Anal. $C_{13}H_{11}N_5O_4S$ (333.32): Calcd: C, 46.84; H, 3.33; N, 21.01; Found: C, 46.72; H, 3.26; N, 21.13.

Cyanoacetic acid N-(6-nitro-2-oxo-2H-chromene-3-sulfonyl)hydrazide (11)

A mixture of 6-nitro-2-oxo-2*H*-chromene-3-sulfonyl chloride (**10**) (2.4 g, 0.01 mol) and 2-cyanoacetic acid hydrazide (1.3 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was stirred for 3 h at room temperature. The solid that formed was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 72%; MP: 154-7° C; IR (KBr, ν_{max}, cm^{-1}) 3325 (br. NH), 2205 (CN), 1710 (C=O), 1685 (C=O), 1596 (C=C), 1386, 1155 (SO_2-N), 1127, 1110 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.25 (1H, s, NH), 8.72 (1H, s, H-4), 8.56 (1H, s, H-5), 8.38 (1H, d, H-7), 7.46 (1H, d, H-8), 7.05 (1H, s, NH), 4.21 ppm (2H, s, CH_2); Anal. $C_{12}H_8N_4O_7S$ (352.28):

Calcd: C, 40.91; H, 2.29; N, 15.90; Found: C, 40.83; H, 2.36; N, 16.05.

5-Amino-1 -(6-nitro-2-oxo-2H-chromene-3-sulfonyl)-1,2-dihydro-pyrazol-3-one (12)

A solution of compound **11** (1.3 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was heated under reflux for 3 h. After cooling, the solid that formed was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 53%; MP: 187-9 °C; IR (KBr, ν_{max}, cm^{-1}) 3410 (NH_2), 3217 (NH), 1712 (C=O), 1676 (C=O), 1553 (C=C), 1373, 1135 (SO_2-N), 1117, 1055 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.51 (1H, s, NH), 8.52 (1H, s, H-4), 8.31 (1H, s, H-5), 7.99 (1H, d, H-7), 7.66 (1H, d, H-8), 6.65 (1H, s, NH), 4.12 ppm (2H, s, CH_2) MS (*m/z*): 352 [M^+]; Anal. $C_{12}H_8N_4O_7S$ (352.28): Calcd: C, 40.91; H, 2.29; N, 15.90; Found: C, 40.85; H, 2.17; N, 16.03.

1-Acetyl-5-amino-4-(6-nitro-2-oxo-2H-chromene-3-sulfonyl)-1,2-dihydropyrazol-3-one (13)

A mixture of **10** (2.4 g, 0.01 mol) and 2-acetyl-2-cyanoacetohydrazide (0.14 g, 0.01mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was stirred for 3 h at room temperature. The solid that formed was filtered off, washed with water; air dried and crystallized from dry ethanol. Yield: 64%; MP: 254 dec. °C; IR (KBr, ν_{max}, cm^{-1}) 3422 (NH_2), 3172 (NH), 1705 (C=O), 1657 (C=O), 1537 (C=C), 1365, 1142 (SO_2-N), 1120, 1035 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.92 (1H, s, NH), 8.93 (1H, s, H-4), 8.53 (1H, s, H-5), 8.41 (1H, d, H-7), 7.50 (1H, d, H-8), 1.77 (2H, s, NH_2) 1.35 ppm (3H, s, CH_3); MS (*m/z*): 394 [M^+]; Anal. $C_{14}H_{10}N_4O_8S$ (394.32): Calcd: C, 42.64; H, 2.56; N, 14.21; Found: C, 42.52; H, 2.41; N, 14.32.

6-Nitro-2-oxo-2*H*-chromene-3-sulfonic acid (5-oxo-4,5-dihydro-1*H*-pyrazol-3-yl)amide (14)

A mixture of **10** (2.4 g, 0.01 mol) and 3-amino-5-pyrazolone (0.99 g, 0.01 mol) in absolute ethanol (20 ml) containing triethylamine (1 ml) was stirred for 3 h at room temperature, the formed precipitate was filtered off, washed with water, air dried and crystallized from dry ethanol. Yield: 81%; MP: 279-81 °C; IR (KBr, ν_{max}, cm^{-1}) 3225 (NH), 3156 (NH), 1712 (C=O), 1677 (C=O), 1601 (C=C), 1385, 1133 (SO_2-N), 1118, 1072 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.92 (1H, s, NH), 8.55 (1H, s, H-4), 8.53 (1H, s, H-5), 8.41 (1H, d, H-7), 7.50 (1H, d, H-8), 4.21 (2H, s, CH_2), 1.77 ppm (1H, s, NH); Anal. $C_{12}H_8N_4O_7S$ (352.28): Calcd: C, 40.91; H, 2.29; N, 15.90; Found: C, 40.83; H, 2.20; N, 15.81.

Synthesis of pyrano(2,3-*c*)pyrazoles 15a-d

A solution of compound **14** (0.35 g, 0.001 mol) and an appropriate arylidene malononitriles (0.001 mol) in dry 1,4-dioxane containing triethylamine (1 ml) was refluxed for 4-6 h. After cooling, the solid that formed was filtered off, washed with water, air dried and crystallized from absolute ethanol.

6-Nitro-2-oxo-2*H*-chromene-3-sulfonic acid (6-amino-5-cyano-4-phenylpyrano(2,3-*c*)pyrazol-3-yl)amide (15a)

Yield: 81%; MP: 141 dec. °C; IR (KBr, ν_{max}, cm^{-1}) 3424 (NH_2), 3212 (NH), 2205 (CN), 1717 (C=O), 1662 (C=C), 1386, 1172 (SO_2-N), 1122, 1101 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.01 (1H, s, NH), 8.71 (1H, s, H-4), 8.31 (1H, s, H-5), 8.20 (1H, d, H-7), 7.21-7.62 (6H, m, Ar-H), 4.57 ppm (2H, s, NH_2); MS (*m/z*): 504 [M^+]; Anal. $C_{22}H_{12}N_6O_7S$ (504.43): Calcd: C, 52.38; H, 2.40; N, 16.66; Found: C, 52.27; H, 2.31; N, 16.52.

6-Nitro-2-oxo-2*H*-chromene-3-sulfonic acid (6-amino-4-(4-chlorophenyl)-5-cyano-pyrano(2,3-*c*)pyrazol-3-yl)amide (15b)

Yield: 53%; MP: 152-4 °C; IR (KBr, ν_{max}, cm^{-1}) 3415 (NH_2), 3165 (NH), 2207 (CN), 1715 (C=O), 1653 (C=C), 1375, 1135 (SO_2-N), 1117, 1053 (C-O-C), 725 (C-Cl); MS (*m/z*): 538/540 [M^+/ M^++2]; Anal. $C_{22}H_{11}ClN_6O_7S$ (538.88): Calcd: C, 49.03; H, 2.06; N, 15.60; Found: C, 48.99; H, 2.14; N, 15.52.

6-Nitro-2-oxo-2*H*-chromene-3-sulfonic acid (6-amino-4-(4-hydroxyphenyl)-5-cyano-pyrano(2,3-*c*)pyrazol -3-yl)amide (15c)

Yield: 59%; MP: 50-2 °C; IR (KBr, ν_{max}, cm^{-1}) 3445 (OH), 3365 (NH_2), 3185 (NH), 2212 (CN), 1710 (C=O), 1623 (C=C), 1382, 1145 (SO_2-N), 1120, 1034 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.25 (1H, s, OH), 9.11 (1H, s, NH), 8.75 (1H, s, H-4), 8.40 (1H, s, H-5), 8.17 (1H, d, H-7), 7.06-7.70 (5H, m, Ar-H), 4.35 ppm (2H, s, NH_2); Anal. $C_{22}H_{12}N_6O_8S$ (520.43): Calcd: C, 50.77; H, 2.32; N, 16.15; Found: C, 50.61; H, 2.25; N, 16.05.

6-Nitro-2-oxo-2*H*-chromene-3-sulfonic acid (6-amino-4-(4-nitrophenyl)-5-cyano-pyrano(2,3-*c*)pyrazol-3-yl)amide (15d)

Yield: 55%; MP: 75-7 °C; IR (KBr, ν_{max}, cm^{-1}) 3386 (NH_2), 3172 (NH), 2195 (CN), 1707 (C=O), 1596 (C=C), 1362, 1133 (SO_2-N), 1118, 1033 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.63-7.42 (8H, m, Ar-H), 7.15 (1H, s, NH), 2.09 ppm (2H, s, NH_2); MS (*m/z*): 549 [M^+]; Anal. $C_{22}H_{11}N_7O_9S$ (549.43): Calcd: C, 48.09; H, 2.02; N, 17.85; Found: C, 48.13; H, 2.16; N, 17.70.

2-(6-Nitro-2-oxo-2*H*-chromene-3-sulfonyl)malononitrile (16)

A mixture of compound **10** (2.89 g, 0.01 mol) and malononitrile (0.6 g, 0.01 mol) in dry ethanol (20 ml) containing triethylamine (1 ml) was heated under reflux for 3 h. The solid that formed was filtered off, washed with water, air dried and crystallized from ethanol. Yield: 86%; MP: 132-4 °C; IR (KBr, ν_{max}, cm^{-1}) 2205, 2197 (CN), 1710 (C=O), 1632 (C=C), 1385, 1175 (SO_2-C), 1110 (C-O-C); Anal. $C_{12}H_5N_3O_6S$ (319.25): Calcd: C, 45.15; H, 1.58; N, 13.16; Found: C, 45.02; H, 1.66; N, 13.09.

3-(3,5-Diamino-4*H*-pyrazole-4-sulfonyl)-6-nitro-chromen-2-one (17)

To a solution of compound **16** (3.19 g, 0.01 mol) in dry ethanol (10 ml) containing few drops of triethylamine, hydrazine hydrate 99% (1 ml, 0.02 mol) was added. The reaction mixture

was refluxed for 6-8 h. After cooling, the reaction mixture was poured onto ice-water (50 ml), and the solid that formed was filtered off, air dried and crystallized from dry ethanol. Yield: 72%; MP: 144 dec. °C; IR (KBr, ν_{max}, cm^{-1}) 3415 (br. NH_2), 1737 (C=O), 1618 (C=N), 1575 (C=C), 1375, 1143 (SO_2-C), 1102 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.91 (1H, s, H-4), 8.61 (1H, s, H-5), 8.43 (1H, d, H-7), 7.46 (1H, d, H-8) 5.52 (2H, s, NH_2), 4.01 (1H, s, CH-pyrazole), 1.95 ppm (2H, s, NH_2); MS (*m/z*): 351 [M^+]; Anal. $C_{12}H_9N_5O_6S$ (351.29): Calcd: C, 41.03; H, 2.58; N, 19.94; Found: C, 41.19; H, 2.47; N, 19.82.

Synthesis of compounds 18a-c

A mixture of compound **16** (3.19 g, 0.01 mol) and urea, thiourea or guanidine hydrochloride (0.01 mol) in dry ethanol (10 ml) containing triethylamine (0.5 ml) was refluxed for 8-10 h. After cooling, the reaction mixture was poured onto ice-water (50 ml) and the solid that formed was filtered off, air dried and crystallized from absolute ethanol.

4,6-Diamino-5-(6-nitro-2-oxo-2*H*-chromene-3-sulfonyl)-5*H*-pyrimidin-2-one (18a)

Yield: 67%; MP: 176 dec °C; IR (KBr, ν_{max}, cm^{-1}) 3425, 3385 (NH_2), 1723, 1695 (C=O), 1620 (C=N), 1587 (C=C), 1365, 1138 (SO_2-C), 1104 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.93 (1H, s, H-4), 8.53 (1H, s, H-5), 8.42 (1H, d, H-7), 7.59 (1H, d, H-8), 6.57 (1H, s, CH pyrimidine), 3.02 (2H, s, NH_2), 1.78 ppm (2H, s, NH_2); MS (*m/z*): 379 [M^+]; Anal. $C_{13}H_9N_5O_7S$ (379.30): Calcd: C, 41.16; H, 2.39; N, 18.46; Found: C, 41.07; H, 2.26; N, 18.35.

3-(4,6-Diamino-2-thioxo-2,5-dihydro-pyrimidine-5-sulfonyl)-6-nitro-chromen-2-one (18b)

Yield: 56%; MP: 119-21 °C; IR (KBr, ν_{max}, cm^{-1}) 3427 (br. NH_2), 1725 (C=O), 1618 (C=N), 1555 (C=C), 1240 (C=S), 1366, 1147 (SO_2-C), 1113 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.87 (1H, s, H-4), 8.42 (1H, s, H-5), 8.37 (1H, d, H-7), 7.57 (1H, d, H-8), 6.40 (1H, s, CH pyrimidine), 3.13 (2H, s, NH_2), 1.91 ppm (2H, s, NH_2); Anal. $C_{13}H_9N_5O_6S_2$ (395.37): Calcd: C, 39.49; H, 2.29; N, 17.71; Found: C, 39.37; H, 2.21; N, 17.62.

3-(4,6-Diamino-2-imino-2,5-dihydro-pyrimidine-5-sulfonyl)-6-nitro-chromen-2-one (18c)

Yield: 51%; MP: 143-5 °C; IR (KBr, ν_{max}, cm^{-1}) 3415 (br. NH_2), 1720 (C=O), 1620 (C=N), 1556 (C=C), 1368, 1157 (SO_2-C), 1107 (C-O-C); MS (*m/z*): 378 [M^+]; Anal. $C_{13}H_{10}N_6O_6S$ (378.32): Calcd: C, 41.27; H, 2.66; N, 22.21; Found: C, 41.19; H, 2.52; N, 22.11.

***N*-(Chlorosulfonyl)-*N*-(2-oxo-2*H*-chromen-6-yl)formamidine (21)**

To a stirred solution of compound **20** (0.94 g, 0.005 mol) in dry benzene (10 ml) was added a solution of chlorosulfonyl isocyanate (0.87 ml, 0.01 mol) in dry benzene (5 ml) at 0-5 °C during 20 min, and the stirring was continued for additional 1 h at the same temperature. The reaction mixture allowed attaining at room temperature for additional 30 min. The reaction mixture was cooled at refrigerator overnight and the solid that formed was filtered off, air dried and crystallized from dry benzene. Yield: 53 %; MP: 224-6 °C; IR (KBr, ν_{max}, cm^{-1}) 3255 (NH), 1733 (C=O), 1618 (C=N), 1563 (C=C), 1385, 1154 (SO_2-N); MS (*m/z*): 286/288 [M^+/ M^++2]; Anal. $C_{10}H_7ClN_2O_4S$ (286.69): Calcd: C, 41.89; H, 2.46; N, 9.77; Found: C, 41.74; H, 2.35; N, 9.61.

1-(*N*-sulfonylchloride)-3-(2-oxo-2*H*-chromen-6-yl) urea (22)

To a stirred solution of **19** (1.6 g, 0.01 mol) in dry benzene (10 ml) was added a solution of chlorosulfonyl isocyanate (0.87 ml, 0.01mol) in dry benzene (5 ml) at 0-5 °C during 20 min, and the stirring was continued for additional 1 h at the same temperature. The reaction mixture allowed attaining at room temperature for additional 30 min. The solid that formed was filtered off; air dried and was used without subsequent cleaning. Yield: 51%; MP: 139-41 °C; IR (KBr, ν_{max}, cm^{-1}) 3225, 3165 (NH), 1735, 1664 (C=O), 1603 (C=C), 1345, 1163 (SO_2), 1109 (C-O-C), 761 (C-Cl); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.95 (1H, s, NH), 8.32 (1H, s, H-5), 8.03 (1H, d, H-7), 7.91 (1H, d, H-4), 7.52 (1H, d, H-8), 7.17 (1H, s, NH), 6.52 ppm (1H, d, H-3); MS (*m/z*): 302/304 [M^+/ M^++2]; Anal. $C_{10}H_7ClN_2O_5S$ (302.69): Calcd: C, 39.68; H, 2.33; N, 9.25; Found: C, 39.55; H, 2.28; N, 9.18.

2,7-Dioxo-pyrano(3,2-*f*)-1,3,4-benzothiazine-5,5-dioxide (23)

To a freshly prepared solution of compound **22** (0.005 mol) in dry benzene (10 ml) aluminum chloride (0.66 g, 0.005 mol) was added at once under stirring at room temperature and then the reaction mixture was refluxed for 30 min. After cooling, the reaction mixture was poured onto ice-water (20 ml), and the solid that formed was filtered off, air dried and crystallized from absolute ethanol. Yield: 30%; MP: 123-5 °C; IR (KBr, ν_{max}, cm^{-1}) 3365 (NH), 1737, 1655 (C=O), 1620 (C=C), 1359, 1171 (SO_2), 1111 (C-O-C); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.33 (1H, d, H-7), 8.01 (1H, d, H-4), 7.62 (1H, d, H-8), 6.46 (1H, d, H-3), 4.23 ppm (2H, s, 2NH); MS (*m/z*): 266 [M^+]; Anal. $C_{10}H_6N_2O_5S$ (266.23): Calcd: C, 45.11; H, 2.27; N, 10.52; Found: C, 45.02; H, 2.20; N, 10.43.

Synthesis of benzenesulfonamides 24a and 24b

A mixture of compound **19** (0.19 g, 0.0005 mol) and 4-bromobenzenesulfonyl chloride or 4-chlorobenzenesulfonyl chloride (0.0005 mol) in dry 1,4-dioxane (10 ml) containing few drops of triethylamine was heated under reflux for 8-10 h. After cooling, the reaction mixture was poured onto cold water (20 ml). The solid that formed was filtered off, air dried and crystallized from 1,4-dioxane.

4-Bromo-*N*-(2-oxo-2*H*-chromen-6-yl)benzenesulfonamide (24a)

Yield: 85%; MP: 160-2 °C; IR (KBr, ν_{max}, cm^{-1}) 3210 (NH), 1722 (C=O), 1605 (C=C), 1364, 1138 (SO_2-N), 1101 (C-O-C), 780 (C-Br); MS (*m/z*): 379/381 [M^+/ M^++2]; Anal.

$C_{15}H_{10}BrNO_4S$ (380.21): Calcd: C, 47.38; H, 2.65; N, 3.68; Found: C, 47.30; H, 2.55; N, 3.59.

4-Chloro-*N*-(2-oxo-2*H*-chromen-6-yl)benzenesulfonamide (24b)

Yield: 87%; MP: 113-5 °C; IR (KBr, ν_{max}, cm^{-1}) 3185 (NH), 1710 (C=O), 1635 (C=C), 1357, 1135 (SO_2-N), 1112 (C-O-C), 752 (C-Cl); MS (*m/z*): 335/337 [M^+/ M^++2]; Anal. $C_{15}H_{10}ClNO_4S$ (335.76): Calcd: C, 53.66; H, 3.00; N, 4.17; Found: C, 53.56; H, 2.94; N, 4.09.

Synthesis of *N*–chlorosulfonyl ureas 25a and 25b

To a stirred solution of compound **24a** or **24b** (0.01 mol) in dry benzene (10 ml) was added a solution of chlorosulfonyl isocyanate (0.87 ml, 0.01 mol) in dry benzene (5 ml) at 0-5 °C during 20 min, and the stirring was continued for additional 1 h at the same temperature. The reaction mixture allowed attaining at room temperature for additional 30 min. The solid that formed was filtered off; air dried and was used without subsequent cleaning.

***N*-(4-Bromobenzene sulfonyl)-*N*-(2-oxo-2*H*-chromen-6-yl)-*N*–chlorosulfonyl urea (25a)**

Yield: 42%; MP: 160-2°C; IR (KBr, ν_{max}, cm^{-1}) 3185 (NH), 1731, 1688 (C=O), 1608 (C=C), 1375, 1153 (SO_2-N), 1115 (C-O-C), 778 (C-Br); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.15 (1H, s, NH), 8.33-7.27 (8H, m, Ar-H), 6.61 ppm (1H, d, H-3); Anal. $C_{16}H_{10}BrClN_2O_7S_2$ (521.75): Calcd: C, 36.83; H, 1.93; N, 5.37; Found: C, 36.75; H, 1.82; N, 5.25.

***N*-(4-Chlorobenzene sulfonyl)-*N*-(2-oxo-2*H*-chromen-6-yl)-*N*–chlorosulfonyl urea (25b)**

Yield: 45%; MP: 157-9 °C; IR (KBr, ν_{max}, cm^{-1}) 3212 (NH), 1735, 1657 (C=O), 1575 (C=C), 1368, 1145 (SO_2-N), 1122 (C-O-C), 775 (C-Cl); ^{1}H NMR (500 MHz, DMSO-*d*6): δ 8.55-7.21 (8H, m, Ar-H), 6.65 (1H, d, H-3), 5.25 ppm (1H, s, NH); Anal. $C_{16}H_{10}Cl_2N_2O_7S_2$ (477.30): Calcd: C, 40.26; H, 2.11; N, 5.87; Found: C, 40.17; H, 2.05; N, 5.77.

Synthesis of pyrano(3,2-*g*)[1,3,4]benzothiazine-9,9-dioxides 26a and 26b

To a freshly prepared solution of compound **25a** or **25b** (0.005 mol) in dry benzene (10 ml) aluminum chloride (0.66 g, 0.005 mol) was added at once under stirring at room temperature and then the reaction mixture was refluxed for 30 min. After cooling, the reaction mixture was poured onto ice-water (20 ml), and the solid that formed was filtered off, air dried and crystallized from absolute ethanol.

2,7-Dioxo-6-(4-bromobenzenesulfonyl)-pyrano(3,2-*g*)[1,3,4]benzothiazine-9,9-dioxide (26a)

Yield: 31%; MP: 172-74 °C; IR (KBr, ν_{max}, cm^{-1}) 3215 (NH), 1732, 1675 (C=O), 1585 (C=C), 1372, 1145 (SO_2-N), 1105 (C-O-C), 775 (C-Br); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.45-7.17 (8H, m, Ar-H), 6.60 ppm (1H, d, H-3); MS (*m/z*): 484/486 [M^+/ M^++2]; Anal. $C_{16}H_9BrN_2O_7S_2$ (485.29): Calcd: C, 39.60; H, 1.87; N, 5.77; Found: C, 39.51; H, 1.73; N, 5.66.

2,7-Dioxo-6-(4-chlorobenzenesulfonyl)-pyrano(3,2-*g*)[1,3,4]benzothiazine-9,9-dioxide (26b)

Yield: 32%; MP: 185-7 °C; IR (KBr, ν_{max}, cm^{-1}) 3302 (NH), 1733, 1683 (C=O), 1567 (C=C), 1355, 1135 (SO_2-N), 1102 (C-O-C), 777 (C-Cl); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.83 (1H, s, NH), 8.54-7.47 (7H, m, Ar-H), 6.45 ppm (1H, d, H-3); MS (*m/z*): 440/442 [M^+/ M^++2]; Anal. $C_{16}H_9ClN_2O_7S_2$ (440.83): Calcd: C, 43.59; H, 2.06; N, 6.35; Found: C, 43.47; H, 2.13; N, 6.22.

Biological assay

Cell line propagation

Hepatocellular carcinoma (HepG2) was purchased from the holding company for biological products and vaccines (VACSERA, Agouza Giza Egypt). Cells were routinely propagated and maintained in RPMI-1640 medium with L-glutamine (Sigma-Aldrich, St Louis, Missouri, USA) and supplemented with fetal calf serum (Sigma-Aldrich, St Louis, Missouri, USA) 10% for growth and 2% for maintenance medium and 1% antibiotic mixture (20 U ml^{-1} of penicillin G sodium and 20 mg ml^{-1} streptomycin sulfate, Gibco™, Thermo Fisher Scientific, Van Allen way carlsbad, CA 92008, USA). Media were changed every 3 days. HepG2 cells were propagated at approximately 80% confluence then trypsinized.

MTT cytotoxicity assay

Cytotoxicity against HepG2 cells was assessed by MTT [3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Bio Basic Canada Inc., Canada) assay (Mosmann 1983). This reaction depends on the mitochondrial reduction of yellow MTT into purple formazan. All the preceding steps were carried out in sterile laminar air flow cabinet Biosafety class II level (Baker, SG403INT; Sanford, ME, USA). Briefly, cells were seeded in 96-well microplates (3 X 10^3cells/well) in 100µl RPMI-1640 culture medium and incubated at 37 °C and 5% CO_2 overnight. The cells were treated and re-incubated for 24 and 48 h. MTT (0.5 mg ml^{-1}) solution was added to each well (100 µl), and the cells were incubated over night until the purple formazan crystals appeared. The medium was discarded; 100 µl of DMSO was added to dissolve the crystals. The optical density (OD) of solubilized formazan was measured at 570 nm using an automatic microplate reader (Bio-Rad Laboratories, model 3350, USA). Dimethyl sulfoxide (DMSO) was the vehicle used for dissolution of testing compound and its final concentration on the cells was less than 0.2%.Results are expressed as percent of control.

Reverse transcription-polymerase chain reaction (RT-PCR) and Real-time Quantitative PCR (q-PCR).

Total RNA was isolated using RNeasy mini Kit (Qiagen, Valencia, CA USA). RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Van Allen way carlsbad, CA 92008, USA) according to

manufacturer. For quantitative real-time PCR, amplification mixtures were prepared using KAPA SYBR_FAST q PCR master mix (KapaBiosystem, Inc., 200 Ballardvale Street Suite 350 Wilmington, USA). Gyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Shao *et al*, 2014) was used as an internal reference gene to normalize the expression of following genes, namely CD105 (Zemel*et al*, 2009), CD44 (Biddle *et al*, 2013), and IGF (Chen *et al*, 2012). The results were expressed as the ratio of reference gene mRNA to target gene mRNA using $2^{-\Delta\Delta Ct}$ method.

Transwell® migration assay

Migration assay was performed in a 24-well transwell® (Sigma-Aldrich, St Louis, Missouri, USA) using polycarbonate membranes with 8-µm pores (Corning® Costar, Cambridge, UK). HepG2 cells were serum-starved by incubating the cells in serum-free media and kept in a 37 °C and 5% CO_2 incubator for 24 h. At a density of 6×105 cells ml^{-1} in 100 µl of serum free medium, HepG2 cells were placed in the upper chamber of the transwell assembly. The lower chamber contained 650 µl of RPMI medium. After incubation at 37 °C and 5% CO_2 for 24 h, the upper surface of the membrane was scraped gently to remove non-migrating cells and washed with phosphate-buffered saline. The membrane was then fixed in 4% paraformaldehyde for 15 min, and stained with hematoxylin and eosin. The cells were then imaged in five fields for each membrane and counted using image J.

MMP-2 activity

MMP-2 activity was measured by RayBio Human MMP-2 ELISA Kit (RayBiotech, Inc., 3607 Parkway Lane suite 200, Norcross, GA 30092, USA), which employs an antibody specific for human MMP-2 coated on a 96-well plate, according to manufacturer. Briefly, Standards and samples are pipetted into the wells and MMP-2 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human MMP-2 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of MMP-2 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Molecular docking

Molecular docking study of coumarin derivatives **4, 5, 8, 12, 13** and **14** were performed by Molecular Operating Environment (MOE) 2008.10 (http://www.chemcomp.com).The PDB code 1HOV was downloaded from protein data bank (http://www. rcsb. org/pdb) (Feng et al, 2002) and prepared for docking process. The co-crystalline ligands were re-docked in the active pockets to validate the docking protocol.

The structure of the target compounds was drawn in ChemDraw Ultra 10.0 (ChemOffice package) and the energy were minimized using the MMFF94x force field until an RMSD (Root-mean-square deviation) of atomic position gradient of (0.01) Kcal $mol^{-1}Å^{-1}$. MMFF94x was reported as the efficient force field for minimizing ligand-protein complexes (Kaminski and Jorgensen 1996)

The docking Algorithm was done by MOE-DOCK default which uses flexible, a rigid technique for posing the molecule inside the cavity. All rotatable bonds of ligands are allowed to undergo free rotation to explore the conformational space inside the rigid receptor binding site.

RESULTS AND DISCUSSIONS

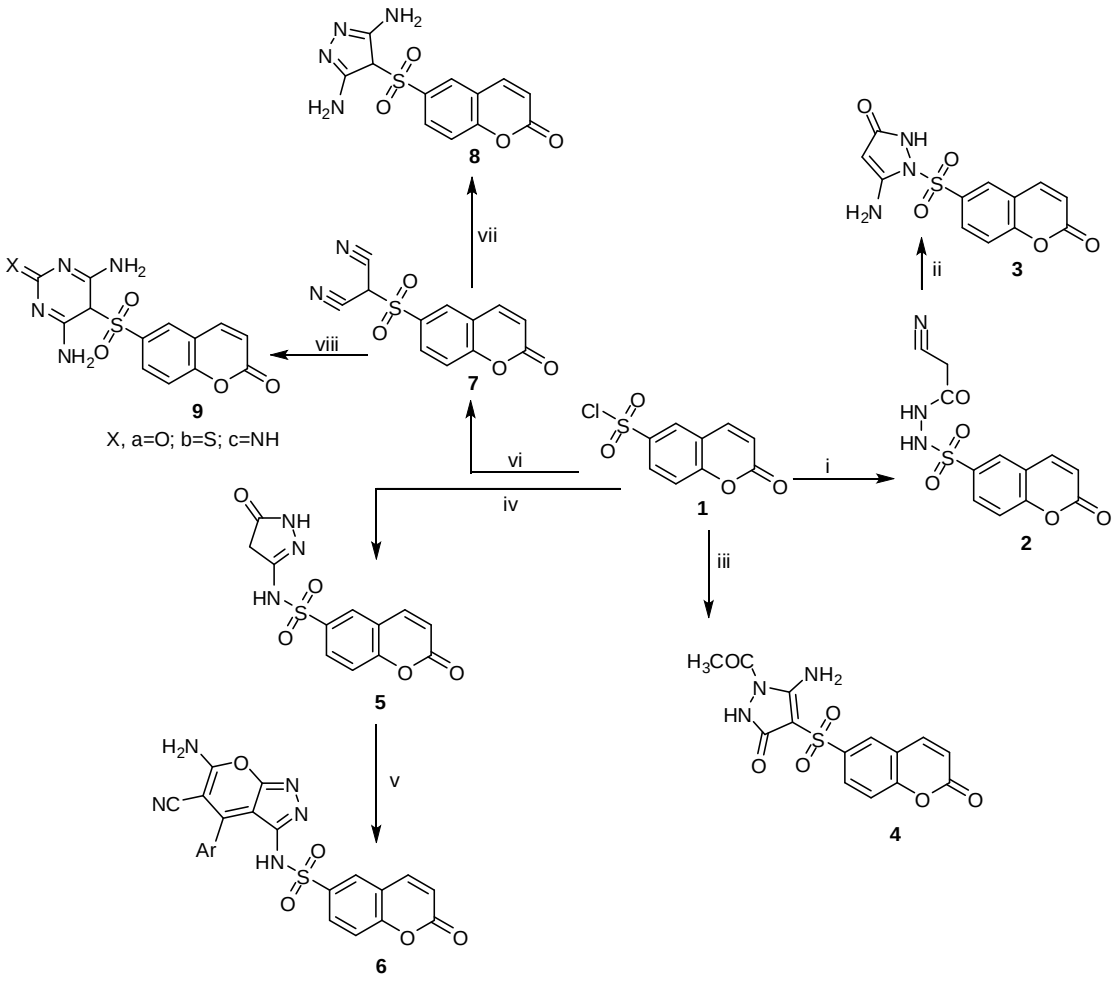
Chemistry

Schemes **1**, **2** and **3** illustrate the reaction routes for the synthesis of the title compounds. Reaction of 2-oxo-2*H*-chromene-6-sulfonyl chloride (**1**) with 2-cyanoacetic acid hydrazide in the presence of few drops of triethylamine under stirring at room temperature led to the formation of *N*'-(2-cyanoacetyl)-2-oxo-2*H*-chromene-6-sulfonohydrazide (**2**). It's ^1H NMR spectrum revealed singlet signals at 9.52 and 7.15 ppm for NH, and 3.15 ppm for CH_2 besides the other signals which located at their position. The IR spectrum of **2** showed absorption bands at 2205 cm^{-1} for CN besides the CO group at 1705 and 1654 cm^{-1}. Intracyclization of the later compound under heating in absolute ethanol containing few drops of triethylamine yielded the aminopyrazolone derivative (**3**) (Scheme 1). It's IR spectrum showed the absence of CN group and showed new absorption bands at cm^{-1} 3410 and 3325 characteristic for NH_2.

On the other hand, reaction of **1** with 2-acetyl-2-cyanoacetohydrazide in absolute ethanol containing few drops of triethylamine yielded, the cyclized 1-acetyl-5-amino-1,2-dihydro-pyrazol-3-one derivative (**4**) (Scheme 1). The IR spectrum of **4** showed the absence of CN group and showed new characteristic absorption bands at 3462, 3346 cm^{-1} for NH_2 beside the absorption band at 3165 for NH.

On the other hand, the base catalyzed reaction of **1** with 3-amino-5-pyrazolinone in dry 1,4-dioxane afforded 2-oxo-2*H*-chromene-6-sulfonic acid(5-oxo-4,5-dihydro-1*H*-pyrazol-3-yl)amide (**5**) (Scheme 1). The reaction of the later compound with some arylidene malononitriles, namely benzylidene malononitrile, 4-chloro, 4-hydroxy, 2-nitrobenzylidene malononitriles in refluxing 1,4-dioxane containing triethylamine led to the formation of the fused system pyrano(2,3-*c*)pyrazole derivatives **6a-d** (Scheme 1).

Moreover, base catalyzed reaction of **1** with malononitrile in dry ethanol afforded 2-(2-oxo-2*H*-chromene-6-sulfonyl)malononitrile (**7**) (Scheme 1). Cyclization of **7** *via* its reaction with hydrazine hydrate, urea, thiourea and/or guanidine hydrochloride in dry ethanol and in the presence of triethylamine led to the formation of the corresponding pyrazole **8** and pyrimidine derivatives **9a-c,** respectively (Scheme 1). In the next bid of the synthesis of new coumarin derivatives, we introduced nitro group at 6-position of coumarin for deactivation the benzene ring of coumarin moiety in order to enhance the electrons density of pyrone ring at 3-position in order to prepare the 6-nitro-2-oxo-2*H*-chromene-3-sulfonyl chloride (**10**) (Abd El-Hafez *et al.*, 1994).



Scheme 1: reagents and conditions: (i) $CNCH_2CONHNH_2$; EtOH; TEA; r.t., (ii) EtOH; TEA; reflux, (iii) $CNCH_2CONHNHCOCH_3$; EtOH; TEA; reflux, (iv) 3-amino-5-pyrazolone; EtOH; TEA; r.t., (v) Ar-CH=C$(CN)_2$; EtOH; TEA; reflux, (vi) $CH_2(CN)_2$; EtOH; TEA; reflux, (vii) $NH_2NH_2.H_2O$; EtOH; TEA, (viii) NH_2CXNH_2; EtOH; TEA.

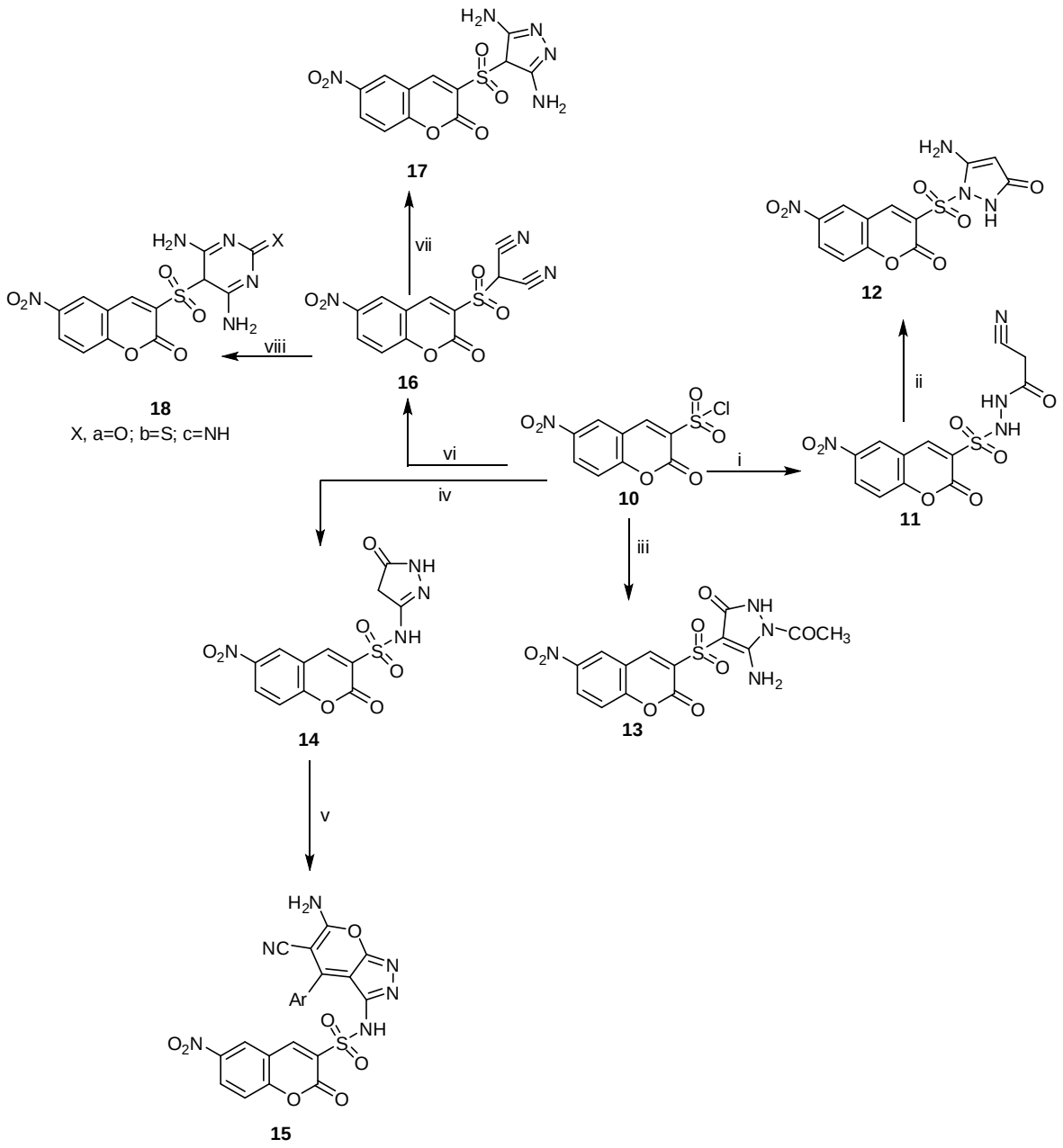
Similarly reaction of **10** with 2-cyanoacetic acid hydrazide in the presence of few drops of triethylamine under stirring at room temperature led to the formation of *N*-(6-nitro-2-oxo-2*H*-chromene-3-sulfonyl)hydrazide (**11**). Cyclocondensation of the later compound under heating in absolute ethanol containing few drops of triethylamine led to the formation of 5-amino-1,2-dihydro-pyrazol-3-one derivative (**12**) (Scheme 2).

Furthermore, cyclocondensation of **10** with 2-acetyl-2-cyanoaceto-hydrazide in the presence of few drops of triethylamine afforded 1-acetyl-5-amino-4-(6-nitro-2-oxo-2*H*-chromene-3-sulfonyl)-1,2-dihydro-pyrazol-3-one (**13**) (Scheme 2). While, reaction of compound **10** with 3-amino-5-pyrazolinone in the presence of triethylamine gave 6-nitro-2-oxo-*2H*-chromene-3-sulfonic acid(5-oxo-4,5-dihydro-*1H*-pyrazol-3-yl)amide (**14**) (Scheme 2). Cyclization of **14** with some arylidene malononitriles, namely benzylidene malononitrile, 4-chloro, 4-hydroxy, 2-nitrobenzylidene malononitriles in refluxing 1,4-dioxane containing triethylamine led to the formation of the fused system, pyrano(2,3-*c*)pyrazole derivatives **15a-d** (Scheme 2).

Moreover, reaction of compound **10** with malononitrile under reflux in dry ethanol containing few drops of triethylamine afforded 2-(6-nitro-2-oxo-2*H*-chromene-3-sulfonyl)malononitrile (**16**) (Scheme 2). Hetero-cyclization of the later compound *via* its reaction with hydrazine hydrate, urea, thiourea and/or guanidine hydrochloride in dry ethanol in the presence of triethylamine led to the formation of the corresponding pyrazole **17** and pyrimidine derivatives **18a-c,** respectively (Scheme 2).

6-Aminocoumarin (**19**) was obtained *via* the reduction of 6-nitrocoumarin using stannous chloride in the presence of tin granules, which under formylation with formic acid yielded the corresponding *N*-(2-oxo-*2H*-chromen-6-yl)formamide (**20**) (Morgan and Micklethwait 1904).

The behavior of chlorosulfonyl isocyanate (CSI) towards aldehyde under stirring in benzene at 0-5 °C results in the formation of (-HC=NSO_2Cl), while, nucleophilic addition reaction of CSI with amine under the previous condition results in the formation of *N*-chlorosulfonyl derivative (-$CONHSO_2Cl$) (Dahr and Murthy 1986).

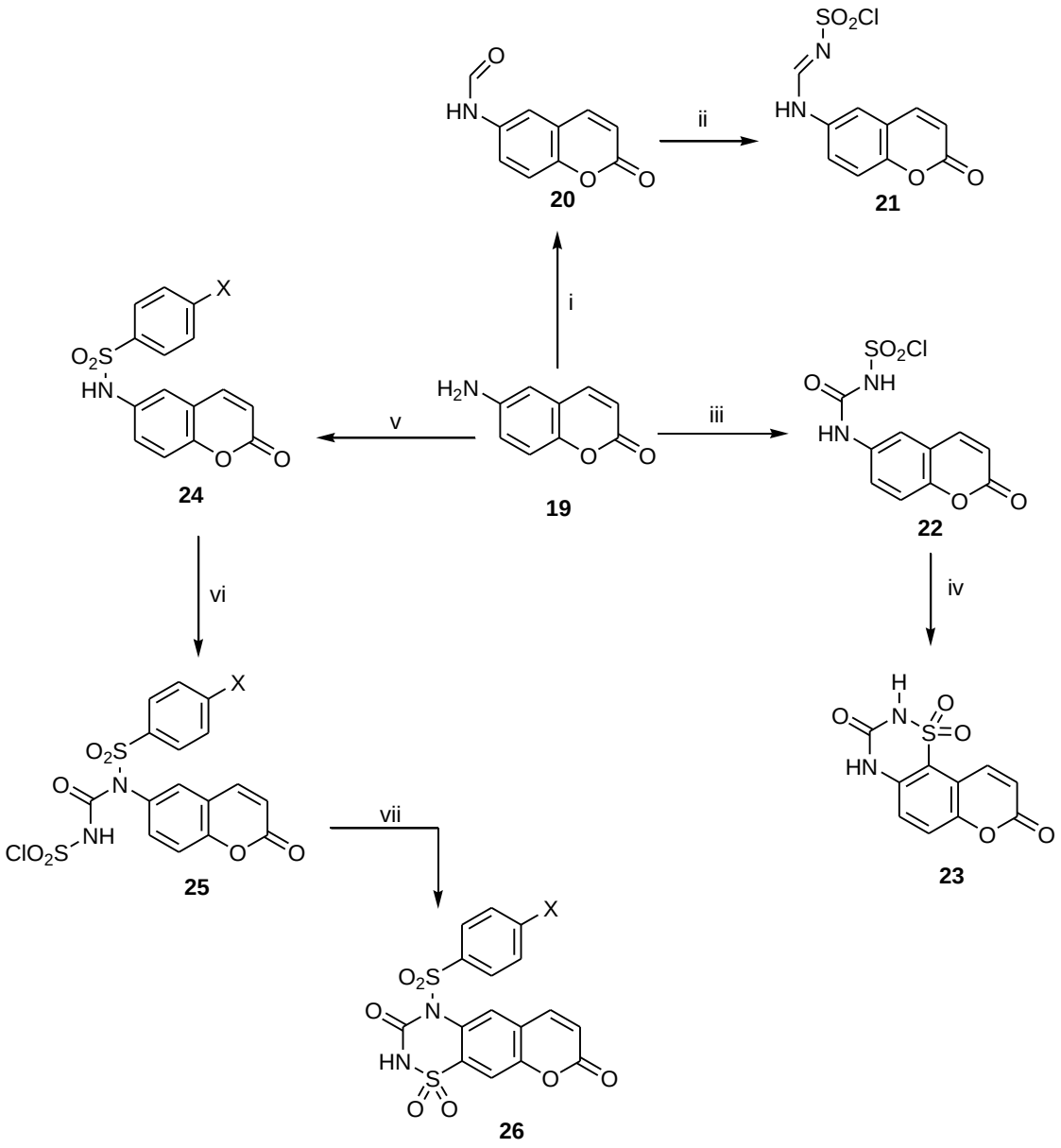


Scheme 2: reagents and conditions: (i) $CNCH_2CONHNH_2$; EtOH; TEA; r.t., (ii) EtOH; TEA; reflux, (iii) $CNCH_2CONHNHCOCH_3$; EtOH; TEA; reflux, (iv) 3-amino-5-pyrazolone; EtOH; TEA; r.t., (v) Ar-CH=C$(CN)_2$; EtOH; TEA; reflux, (vi) $CH_2(CN)_2$; EtOH; TEA; reflux, (vii) $NH_2NH_2.H_2O$; EtOH; TEA, (viii) NH_2CXNH_2; EtOH; TEA.

In the present work and under the previous conditions, reaction of aldehyde **20** with chlorosulfonyl isocyanate (CSI) in dry benzene at 0-5 °C afforded the new *N*-(chlorosulfonyl)-*N*-((2-oxo-2*H*-chromen-6-yl)formamidine (**21**) (Scheme 3). Compound **21** give positive sulfur and chlorine tests as a chemical evidence, besides IR and mass spectrum.

On the other hand, the reaction of 6-amino coumarin **(19)** with chlorosulfonyl isocyanate in dry benzene at 0-5 °C gives 1-(*N*-sulfonylchloride)-3-(2-oxo-2*H*-chromen-6-yl) urea (**22**), which on cyclization with an aluminum chloride (Lewis acid) gave the corresponding 2,7-dioxo pyrano(3,2-*f*)[1,3,4]benzothiazine-5,5-dioxide (**23**) (Scheme 3).

In order to obtain new *N*-sulfonamide derivatives, compound **19** allowed to react with 4-bromo and 4-chloro benzene sulfonyl chloride under reflux in dry 1,4-dioxane containing few drops of triethylamine to give compounds **24a,b**, which upon reaction with chlorosulfonyl isocyanate in dry benzene at 0-5 °C yielded *N*-sulfonylchloride derivatives **25a,b**. The freshly prepared **25a,b** were cyclized using aluminium chloride to give the corresponding thiadiazine derivatives **26a,b** (Scheme 3)



24, **25**, **26**, X, a=Br; b=Cl

Scheme 3: reagents and conditions: (1) HCOOH; reflux, (Morgan and Micklethwait 1904), (ii) CSI; dry benzene; 0-5 °C; stirring, (iii) CSI; dry benzene; 0-5 °C, (iv) $AlCl_3$, dry benzene; reflux, (v) $XC_6H_4SO_2Cl$; 1,4-dioxan; TEA, (vi) CSI; dry benzene; 0-5 °C; stirring, (vii) $AlCl_3$, dry benzene; reflux

Biological activity results

The non-cytotoxic tested compounds **4**, **5**, **8**, **12**, **13** and **14** against hepatocellular carcinoma cells (HepG2) (Figure 1) significantly inhibited MMP-2 activity at p value < 0.001 except **8** and **12** at p value < 0.05 as a percent of control. All compounds exhibited high anti-migratory effect as revealed by transwel migration assay (number of migrated cells relative to control was 3.7, 3.3, 4.7, 3.4, 3.7, and 4.2 for compounds **4**, **5**, **8**, **12**, **13**, **14**, respectively (Figures 2a,b). Gene expression of IGF was not affected by any of the selected compounds (Figure 3). CD105 which is a surface marker was up regulated with all compounds except compound **4** which down regulated CD105. Compound **5** and **8** up-regulated the surface marker CD44, while other compounds had no effect on CD44 expression (Figure 4)

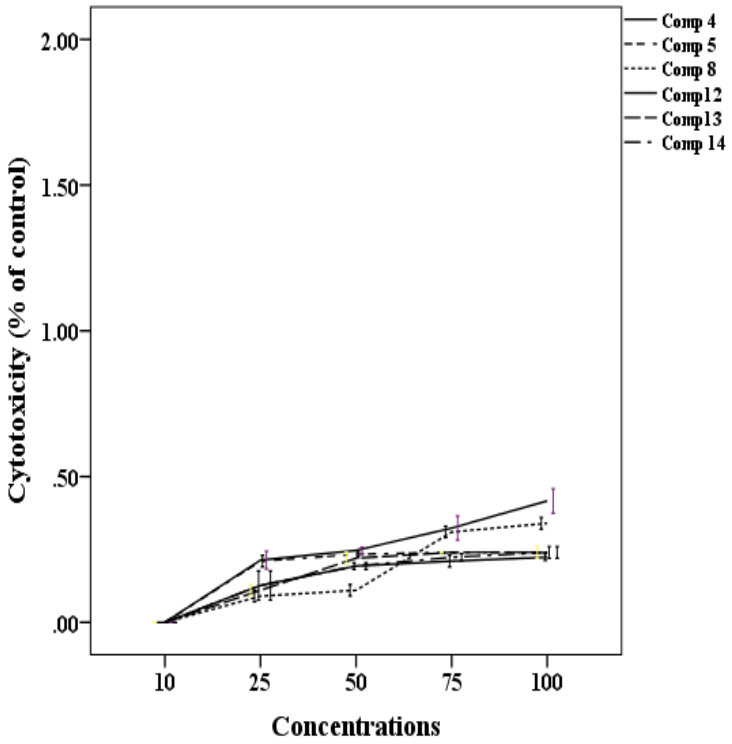


Fig. 1: Cytotoxic activity of the tested compounds against human hepatocellular carcinoma (HepG2) treated at various concentrations for 48 h. Data were expressed as percent of control ± SE (n=3). Significantly different by Tukey's test ($p<0.05$).

Biological activity discussion

Anti-angiogenic therapies are promising for the treatment of cancer. Tumor metastasis is also regulated by angiogenes. In combination therapies, the efficacy of chemotherapy is enhanced by anti-angiogenic drug. The resistance to conventional cytotoxic therapeutics, emphasize the need for efforts to develop non-cytotoxic targeted molecular therapies directed against the pathways involved in the angiogenesis.

Coumarin molecules can be utilized as lead compounds to develop potential nontoxic angiogenesis inhibitors (Nam*et al*., 2002; Lee *et al* 2006). Generally, cells have been proposed to employ either protease-dependent (MMP-dependent) or protease-independent (MMP-independent) modes for migration and invasion (Wolf and Friedl, 2011). Many genes, proteins and pathways have been identified as potential targets for anti-angiogenic agents CD44; a transmembrane proteoglycan known to be expressed in most human cancers has been investigated as a therapeutic drug delivery target. CD105 (endoglin) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells (EC).

All the tested synthesized compounds showed non-cytotoxic effects against hepatocellular carcinoma cells (HepG2) (Figure 1), and exhibited high anti-migratory effect as revealed by transwel migration assay (Figure 2a, b). They also significantly inhibited MMP-2 activity except compounds **8** and **12**. Gene expression of IGF was not affected by any of selected compounds. CD105 which is a surface marker was not involved in their anti-migrator activity where it was up-regulated with all compounds. In case of compound **4** the anti-migrator activity was mediated by down regulation of CD105. The surface marker CD44 was not involved in the anti-migratory activity induced by compound **5** and **8** where it was up-regulated, while other compounds had no effect on CD44 expression.

Compound **4** considered a promising anti-angiogenic agent where it exhibited MMP-dependent anti-migratory activity and down regulated CD105; however it has no effect on CD44.

The anti-migratory activity of the compounds which accompanied with up-regulation of CD44 is concomitant with that induced by docetaxel (DTX) treatment irrespective of the tumor type (Goldman *et al*., 2015).

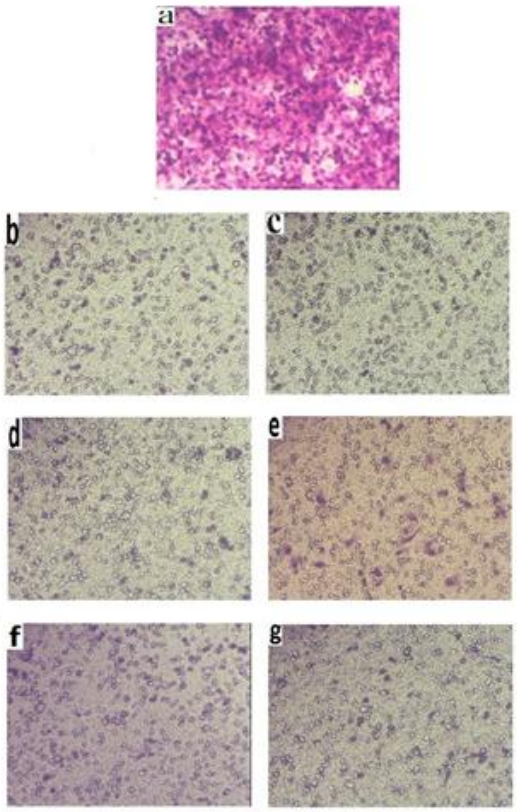


Figure 2a

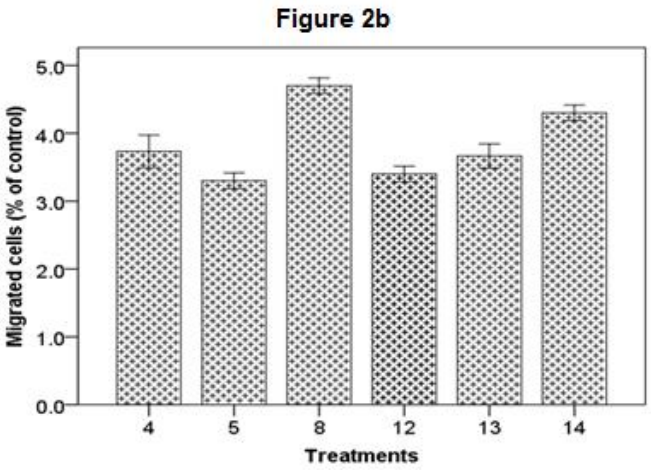


Fig. 2: Migration of human hepatocellular carcinoma (HepG2) in response to treatment with the tested compounds. **(A)** Appearance of HepG2 cells (haematoxylin-eosine stained) on the underside of the membrane in the migration assay control. a: control, b: compound **4** ,c: compound **5**, d: compound **8**, e: compound **12**, f: compound **13**, g: compound **14**. **(B)** Migrated cells percent in response to different treatments. Significantly different by Tukey's test ($p<0.05$)

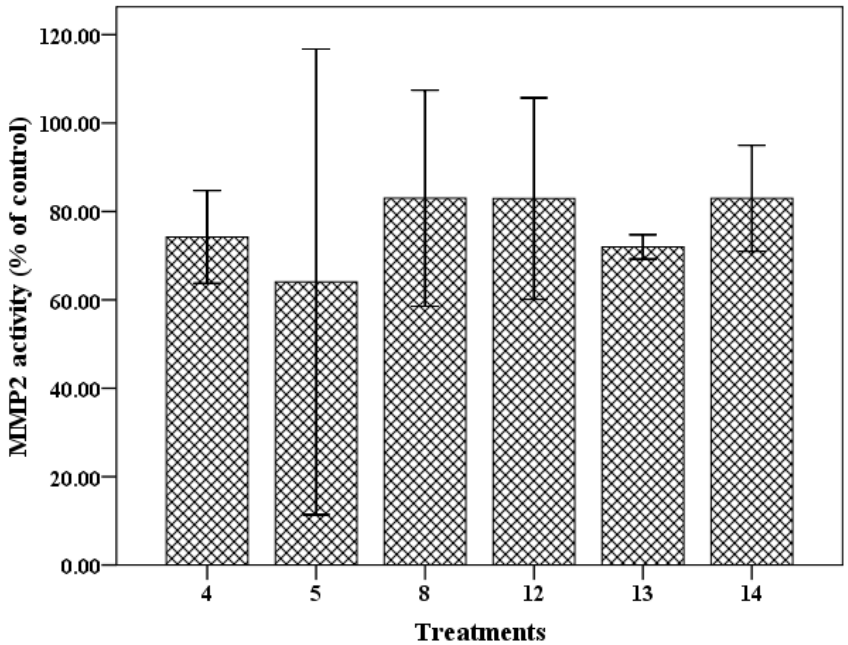


Fig. 3: Matrix metalloproteinase 2 of human hepatocellular carcinoma (HepG2) in response to treatment with the tested compounds. MMP-2 activity represented as percent of control. Significantly different by Tukey's test ($p<0.05$)

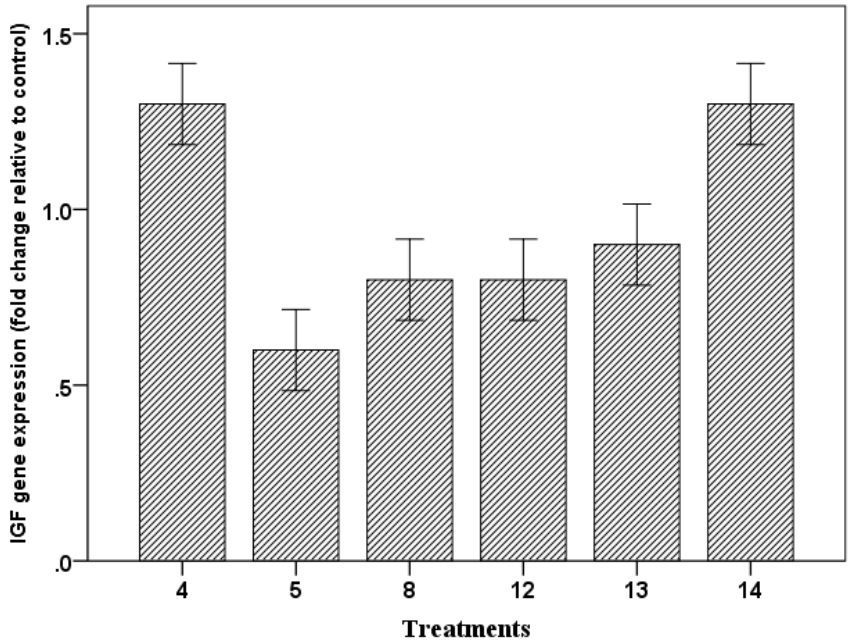


Fig. 4: Gene expression of insulin–like growth factor of human hepatocellular carcinoma (HepG2) in response to treatment with the tested compounds. Data was represented as fold change relatively to control. Significantly different by Tukey's test ($p<0.05$)

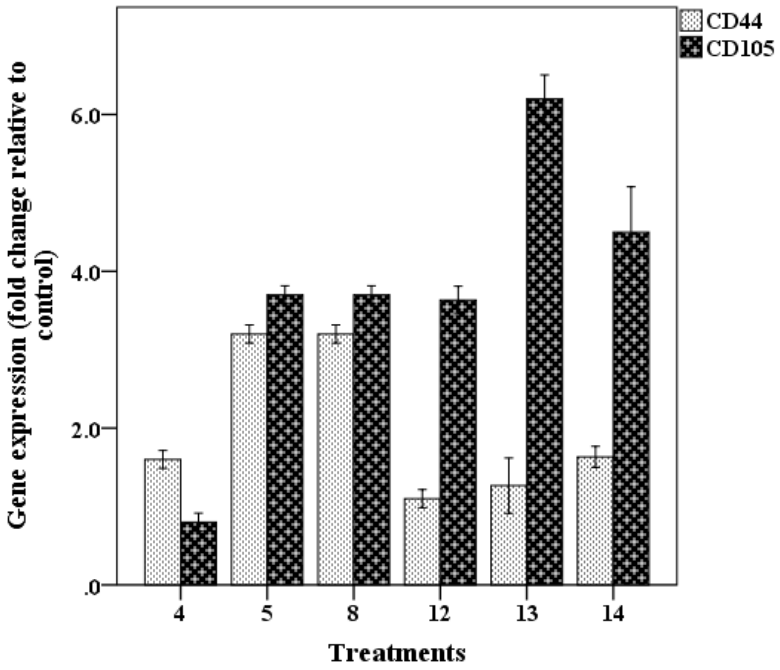


Fig. 5: Gene expression of surface markers CD44 and CD105 of human hepatocellular carcinoma (HepG2) in response to treatment with the tested compounds. Data was represented as fold change relatively to control. Significantly different by Tukey's test ($p<0.05$)

Molecular docking study

MMP-2, a zinc-containing enzyme, plays an important role in cancer, by stimulating tumors growth, angiogenesisand metastasis, through its involvement in the degradation of extracellular matrix (Zapico et al., 2011) MMP-2 has been considered for many years an important target for the design of anticancer agents. For the coumarin derivatives **4, 5**, **8**, **12, 13** and **14** we first evaluated the suitability of these compounds to act as MMP-2 inhibitors by means of docking technique. There are not many experimental 3D structures of MMP-2 available on the protein data bank, PDB 1HOV being the only complex among MMP-2 catalytic domain with an inhibitor, hydroxamate **I52.** PDB 1HOV is an NMR structure composed of 11 models, and the superimposition of all of them showed no relevant changes around

the ligand binding region. The previous studies of the binding mode of a set of putative MMP-2 inhibitors, including **I52**, showed no difference in the docking results performed on the 11 models, so we considered only model 1 to carry out the docking studies **(**Garcìa, 2007**)**. The docking result showed that (Table 2), all docked compounds exhibit better docking score and good fitting inside the active side of MMP-2 (PDB: 1HOV) *via* formation of hydrogen bonds and coordination bonds with catalytic Zn^{++} ion compare to co-crystalline ligand **I52**.

It has been observed that nitro coumarin derivatives **12, 13** and **14** were exhibited better docking score (-28.17 to -18.81 kJ mol^{-1}) than coumarin derivatives **4**, **5** and **8** (-16.22 to -13.82kJ mol^{-1}), and also higher than docking score of **I52** (-18.18 kJ mol^{-1}). Also the presence of nitro group enhance the ability of compounds to coordinate with Zn^{++} ion for example in case of compound **14,** forms two coordination bonds with catalytic Zn^{++} ion *via* two oxygen atoms of SO_2 group and coumarin moiety while in case of compound **5** no interaction occur between the predicted binding pose and Zn^{++} ion (Figures **6** and **7**). On the other hand, it has been noticed that the anti-angogenic activity of the nitro compounds designed from postulation of molecular docking was not significant *in-vitro*. Only compound 4 with *N*-acetylpyrazolone substitution at the 6-position of sulfonyl coumarin showed a promising anti-angiogenic activity, where exhibited good docking score of -16.22 compare to the MMP-2 inhibitor (**I52**) of -18.18 $kJmol^{-1}$ (Table 2). Also, compound **4** showed better binding interaction with the active site of 1HOV *via* formation of a) one hydrogen bond acceptor between oxygen atom of $COCH_3$ group and NH of Leu83 (2.58Å), b) one hydrogen bond donor between NH of acetyl pyrazolone ring and oxygen atom of Glu121 (1.45 Å), c) coordination bond between oxygen atom of pyrazolone ring with catalytic Zn^{++} ion (Table 2, Figures 8a, b).

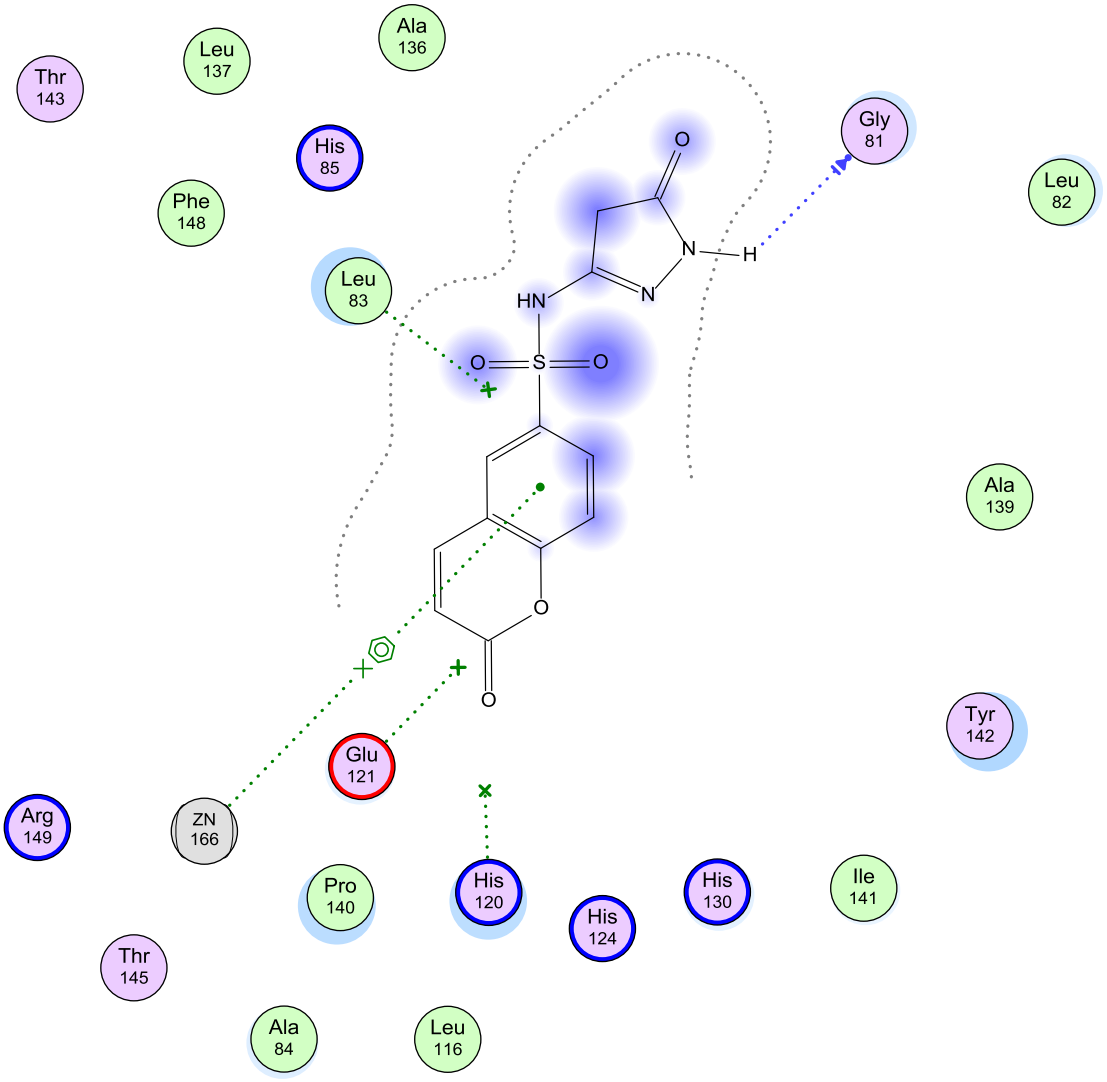


Fig. 6: The 2D depiction of the docked conformation of **5** into active side of MMP-2 (PDB ID: 1HOV).

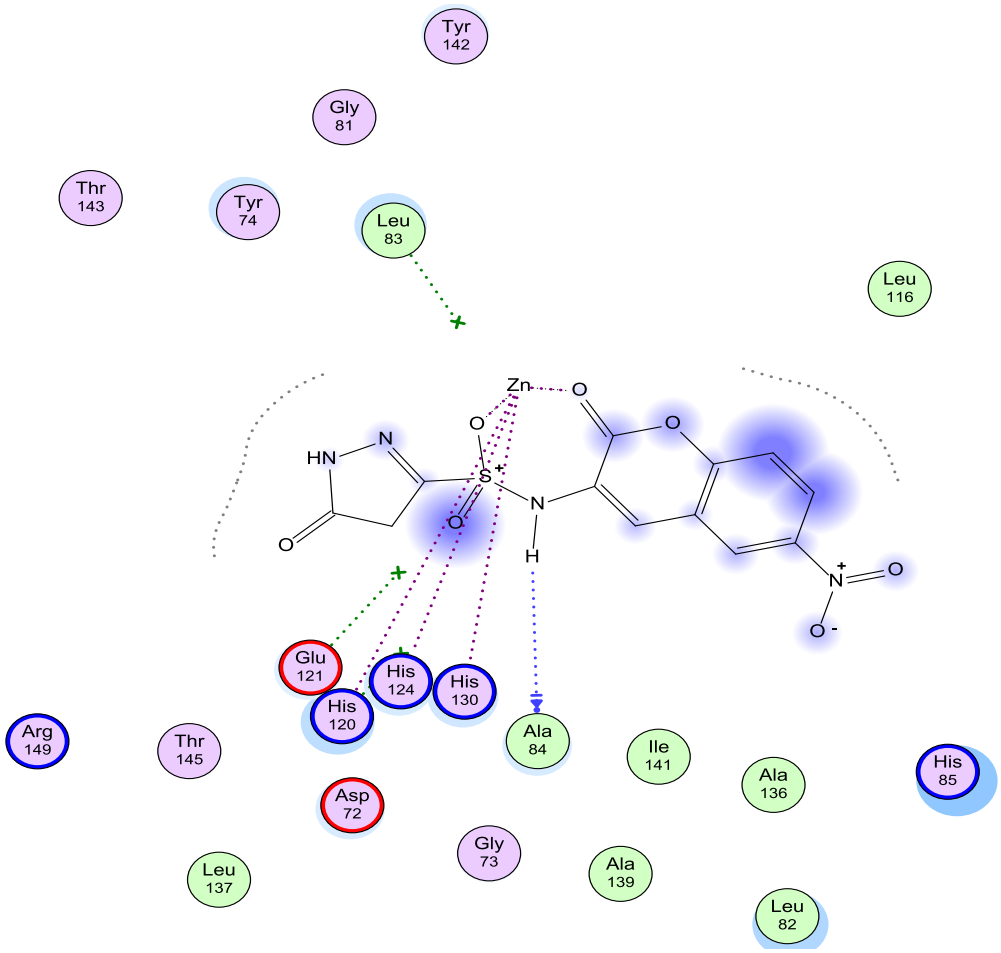


Fig. 7: The 2D depiction of the docked conformation of **14** into active side of MMP-2 (PDB ID: 1HOV).

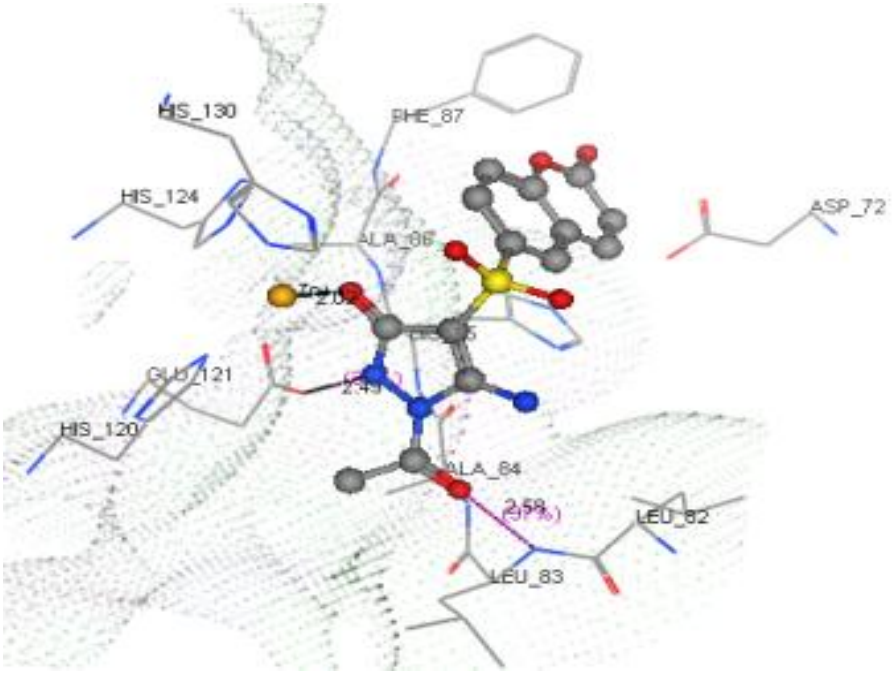


Fig. 8a: The 3D depiction of the docked conformation of **5** into active side of MMP-2 (PDB ID: 1HOV).

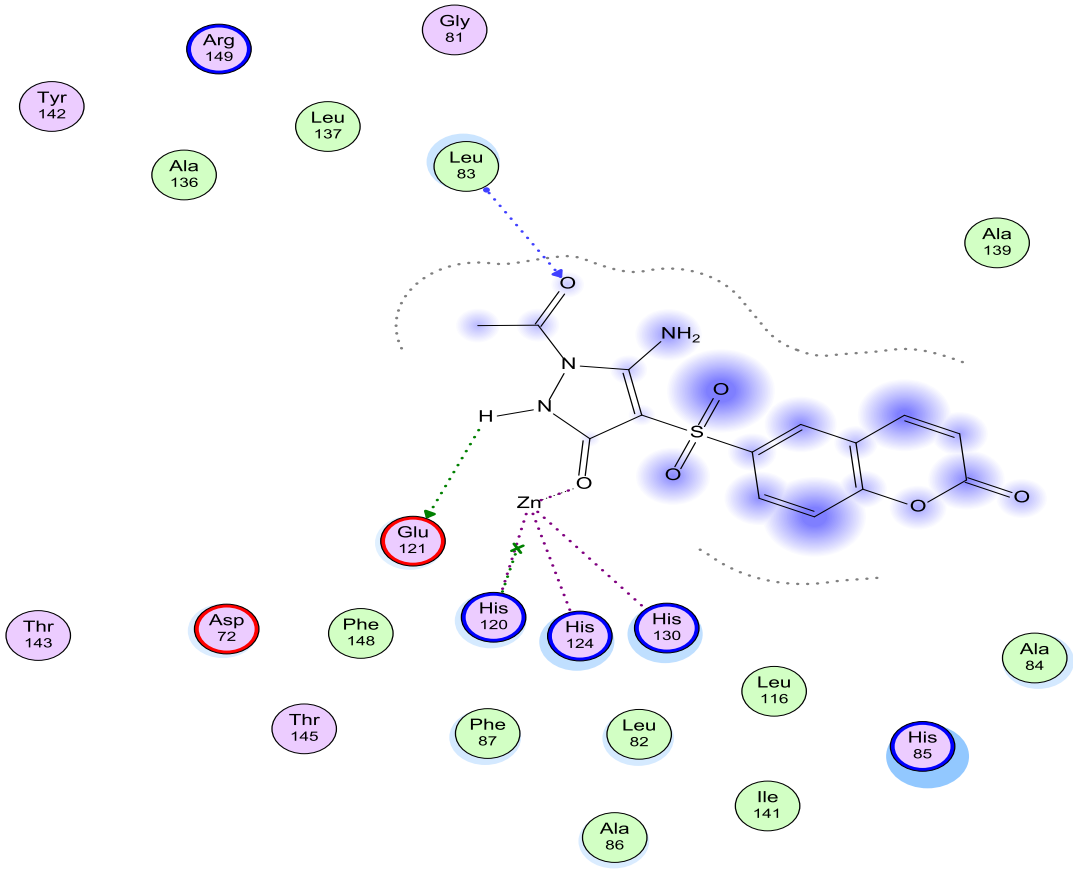


Fig. 8b: The 2D depiction of the docked conformation of **5** into active side of MMP-2 (PDB ID: 1HOV).

Table 1: List of primers genes.

Gene	Forward primer	Reverse primer
GAPDH	5′-ACCCACTCCTCCACCTTTGAC-3′	5′-TGTTGCTGTAGCCAAATTCGTT-3
CD105	5′- CTCTGCTGCTGAGCTGAATG-3	5′-GATCTGCATGTTGTGGTTGG-3
CD44	5’-AGAAGGTGTGGGCAGAAGAA-3’	5’-AAATGCACCATTTCCTGAGA-3’
IGF	5′- GCAATGGGAAAAATCAGCAG-3′	5′-GAGGAGGACATGGTGTGCA-3′

Table 2: Docking results of the most active compounds which docked with MMP-2 (PDB ID: 1HOV).

Compd. No	Mol. Dock Score (kJ mol^{-1})	Type of bond	Atom of ligand involved	Involved atom of amino acid	Length of bond (Å)
I52	-18.18 Rmsd(1.04)	H-don.	OH	N His120	3.41
		H-don.	HN	O Glu121	1.99
		H-acc.	O of SO_2	N Leu83	3.05
		Ionic	two hydroxamate oxygen atoms	ZN^{++}	2.13&2.08
4	-16.22	H-acc.	O of $COCH_3$	N of Leu83	2.58
		H-don.	NH of acetyl pyrazolone	O of Glu121	1.45
		Ionic	O of pyrazolone	Zn^{++}	2.03
5	-14.41	H-don.	NH of pyrazolone	O of Gly81	1.65
8	-13.82	H-don.	NH of pyrazole	O of Ala84	1.39
		H-don.	NH of pyrazole	O of Glu121	1.35
		H-acc.	O of SO_2	NH of Leu83	2.89
		Ionic	Two nitrogen atoms of pyrazole	Zn^{++}	2.62 & 2.10
12	-28.17	H-don.	NH of pyrazolone	O of Glu121	1.62
		Ionic	O of SO_2 & O of coumarin moiety	Zn^{++}	2.67 & 2.04
13	-21.42	Ionic	O of $COCH_3$	Zn^{++}	2.15
14	-18.81	H-don.	NH of SO_2NH	O of Ala84	2.06
		Ionic	O of SO_2 and O of coumarin moiety	Zn^{++}	2.92 & 1.99

don:- donator; acc:- acceptor

CONCLUSION

Our study aimed to synthesize new non-cytotoxic sulfonyl coumarin derivatives against hepatocellular carcinoma cells (HepG2) for further test as anti-angiogenic agents using migration assay and MMP-2 activity by ELISA. Collectively, our results indicate that, coumarin molecules **4**, **5**, **8**, **13** and **14** can be utilized as lead compounds to develop potential non-toxic angiogenesis inhibitors and small molecular ligands to target (HepG2), which was in concomitant with molecular docking results. 1-Acetyl-5-amino-4-(2-oxo-2H-chromene-6-sulfonyl)-1,2-dihydro-pyrazol-3-one (**4**) considered a promising anti-angiogenic agent, where it exhibited MMP-dependent anti-migratory activity and down regulated CD105.

AKNOLEDGMENT

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Conflict of Interests: There are no conflicts of interest.

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