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SÍNTESE E ATIVIDADE ANTITUMORAL DE NOVAS CUMARINAS MULTIFUNCIONAIS

SYNTHESIS AND ANTITUMOR ACTIVITY OF NEW MULTIFUNCTIONAL COUMARINS

تصنيع وفحص النشاط المضاد للورم لمركبات جديدة من الكومارينات المتعددة الوظائف

BASHIR, Moath Kahtan [1]; MUSTAFA, Yasser Fakri [2*]; OGLAH, Mahmood Khudhayer [3];

[1, 2, 3] Pharmaceutical Chemistry Department, College of Pharmacy, Mosul University-41002, Nineveh, Iraq.

** Corresponding author*
E-mail: Dr.yassermustafa@uomosul.edu.iq

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RESUMO

O câncer constitui uma das mais graves ameaças à saúde pública em todo o mundo. É imperativo sintetizar novos compostos e explorar sua atividade antitumoral para encontrar uma resolução potencial para este problema de saúde. A síntese de novos scaffolds e a avaliação de sua atividade antitumoral é uma abordagem relevante para o combate ao desenvolvimento do câncer. As cumarinas podem apresentar diversas atividades biológicas, sendo uma delas a atividade antitumoral. Este estudo teve como objetivo sintetizar novas cumarinas enxertando seus precursores nas aminas aromáticas via formação de base de Schiff e avaliando sua atividade antitumoral introdutória. Novas cumarinas multifuncionais (MC1-MC9) foram preparadas integrando uma cumarina funcionalizada com diferentes derivados de toluidina por meio de uma ligação à base de Schiff. A caracterização espectral inspirada nas espectroscopias FTIR, ^{1}H- e ^{13}C- NMR estabeleceu as estruturas químicas dos produtos sintetizados. A atividade antitumoral foi explorada in vitro contra quatro linhagens de câncer humano dominante, incluindo HeLa, SKG, MCF-7 e AMN3. Os resultados adquiridos do ensaio de viabilidade celular inspecionado pela aplicação de corante MTT revelaram que as cumarinas multifuncionais sintetizadas, particularmente MC3, têm uma atividade promissora. Pode-se concluir que para as cumarinas sintetizadas foi observada tendência semelhante de atividade contra as linhagens de células de teste, sendo a melhor ação contra MCF-7 e, pelo menos, uma contra AMN3. Este estudo não apenas fornece uma nova estrutura de uma atividade antitumoral significativa, mas também fornece alguns insights sobre sua relação estrutura-atividade.

Palavras-Chave: *Base de Schiff, cumarina, atividade antitumoral, ensaio de MTT.*

ABSTRACT

Cancer constitutes one of the most severe public health menaces worldwide. It is imperative to synthesize new compounds and explore their antitumor activity to find a potential resolution to this health problem. Synthesis of new scaffolds and evaluating their antitumor activity is a relevant approach for combating cancer development. Coumarins can exhibit diverse biological activities, and one of these is the antitumor activity. This study aimed to synthesize new coumarins by grafting their precursors to the aromatic amines via Schiff base formation and evaluating their introductory antitumor activity. New multifunctional coumarins (MC1-MC9) were prepared by integrating a functionalized coumarin with different toluidine derivatives via a Schiff-base linkage. Spectral characterization inspired by FTIR, ^{1}H- and ^{13}C- NMR spectroscopies has established the chemical structures of the synthesized products. The antitumor activity was explored *in vitro* versus four dominant human cancer lines, including HeLa, SKG, MCF-7, and AMN3. The outcomes acquired from the cell viability assay inspected by applying MTT dye have revealed that the synthesized multifunctional coumarins, particularly MC3, have a hopeful activity. It can be concluded that a similar trend of activity against the test cell lines was observed for the synthesized coumarins, with the best action being versus MCF-7 and the least one versus AMN3. This study not only affords a new scaffold of a significant antitumor activity but also provides some insights into its structure-activity relationship.

Keywords: *Schiff-base, coumarin, antitumor activity, MTT assay.*

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الخلاصة

يشكل السرطان أحد أخطر تهديدات الصحة العامة في جميع أنحاء العالم. من الضروري تصنيع مركبات جديدة واستكشاف نشاطها المضاد للأورام لإيجاد حل محتمل لهذه المشكلة الصحية. يعد تركيب السقالات الجديدة وتقييم نشاطها المضاد للأورام نهجًا مناسبًا لمكافحة تطور السرطان. يمكن أن تظهر الكومارينات أنشطة بيولوجية متنوعة ، وأحد هذه الأنشطة هو النشاط المضاد للأورام. هدفت هذه الدراسة إلى تصنيع كومارين جديدة عن طريق تطعيم سلائفها للأمينات العطرية عن طريق تكوين قاعدة شيف وتقييم نشاطها التمهيدي المضاد للأورام. تم تحضير الكومارينات الجديدة المتعددة الوظائف (MC1-MC9) من خلال دمج الكومارين الوظيفي مع مشتقات التولويدين المختلفة عبر ارتباطها بقاعدة شيف. إن التوصيف الطيفي المستوحى من مطياف الاشعة تحت الحمراء والرنين النووي المغناطيسي للبروتون وللكاربون المشع قد أنشأ الهياكل الكيميائية للمنتجات المركبة. تم استكشاف النشاط المضاد للورم في المختبر مقابل أربعة خطوط سرطانية بشرية سائدة ، بما في ذلك سرطان عنق الرحم و سرطان المرئ و سرطان الثدي وسرطان الغدة الثديية الفئران. أظهرت النتائج المكتسبة من اختبار قابلية الخلية للفحص عن طريق تطبيق صبغة التسرطن أن الكومارينات المصنعة، وخاصة مركب MC3 ، له نشاط يبعث على الأمل. يمكن أن نستنتج أنه لوحظ وجود اتجاه مماثل للنشاط ضد خطوط خلايا الاختبار بالنسبة للكومارينات المُصنَّعة، مع أفضلية لخلايا MCF-7 والأقل مقابل AMN3 . لا توفر هذه الدراسة دعامة جديدة للنشاط المضاد للأورام المهمة فحسب ، بل توفر أيضًا بعض الأفكار حول علاقة الهيكل الكيميائي بالنشاط المضاد للسرطان.

الكلمات الافتتاحية: قاعدة شيف ، كومارين ، نشاط مضاد للورم ، صبغة فحص التسرطن

1. INTRODUCTION:

Cancer constitutes one of the most serious public health menaces worldwide. The rate of its incidence is highly elevated in almost all parts of the world since 1990 (Fitzmaurice *et al.*, 2015). The effective prevention and treatment of different kinds of cancer are hard to achieve despite discovering and developing many experimental antitumor agents from natural (Rahimi Khoigani *et al.*, 2017) or synthetic origins (Mustafa, 2019). Therefore, it is still a fundamental demand to synthesize new chemical entities and explore their antitumor activity to find a potential resolution to this mysterious health problem (Bashir *et al.*, 2020).

Coumarin is a charming chemical nucleus and alongside its natural and synthetic derivatives comprise an important oxygen-containing heterocycle class. Coumarins as bioactive agents can exhibit diverse biological activities, which include the antibacterial (Kumar *et al.*, 2015; Al Zoubi *et al.*, 2018; Gonelimali *et al.*, 2018), antifungal (Medimagh-Saidana *et al.*, 2015), anticholinesterase (Cakmak and Gülçin, 2019), antitumor (Jing Lia, Fei Yua, Yi Chena, 2015; Detsi *et al.*, 2017; Yao *et al.*, 2017; Haq *et al.*, 2019), anti-inflammatory (Srikrishna *et al.*, 2016), antioxidant (Chen, 2016; Pasciu *et al.*, 2019; Alfahad *et al.*, 2020), antidiabetic (Pisoschi and Pop, 2015; Li *et al.*, 2017; Forni *et al.*, 2019; Iheagwam *et al.*, 2019), and many other important effects (Stefanachi *et al.*, 2018). Although there are many synthetic routes for coumarins preparation, Pechmann condensation, and its recent improvements remain the most utilized technique (Dandriyal *et al.*, 2016; Jung *et al.*, 2018; Teran *et al.*, 2019).

Schiff bases, or as named azomethines comprise the functional group that results from the direct condensation of a carbonyl compound with primary amine (Kajal *et al.*, 2013). In addition to its soft formation, Schiff base can be considered a versatile pharmacophore that exhibits various biological and medicinal activities. Example activities may include anti-inflammatory, anticancer, analgesic, antioxidant, antimicrobial, anticonvulsant, antidepressant, and antitubercular effects (Hameed *et al.*, 2017). In this context, Schiff bases of primary aromatic amine substituted with the electron-donation group have played an essential role in regulating and enhancing the activities mentioned above (Al Zoubi *et al.*, 2018).

This study aimed to synthesize new coumarins by grafting their precursors to the aromatic amines via Schiff base formation and evaluating their introductory antitumor activity utilizing MTT test versus four human cancer cells lines named HeLa, SKG, MCF-7, and AMN3.

2. MATERIALS AND METHODS:

2.1. Reagents and chemicals

Chemicals, solvents, and reagents applied in this study were obtained from Scharlau, Haihang, Sigma-Aldrich, Bio-World, and other documented international companies. These chemicals and reagents included concentrated H_2SO_4, citric acid, m-guaiacol, NaOH, lithium tri-*tert*-butoxyaluminum hydride, acetaldehyde, propionaldehyde, butyraldehyde, 2-methyltoluidine, 3-methyltoluidine, and 4-methyltoluidine.

2.2. Thin-layer chromatography (TLC)

The ascending TLC technique was used to establish the purity of the synthetic products using pre-coated silica gel plates (GF254 type 60, Merck, Germany). The spots were eluted on the chromatograms using $CHCl_3$: methanol (5:1) as an eluent.

2.3. Melting point (mp)

The electrochemical CIA 9300 instrument was utilized for the examination of the melting points of synthesized products through an open-capillary method, and they are uncorrected.

2.4. Fourier transforms infrared (FTIR*)*

The FTIR spectra of the synthetic products were recorded by using Bruker-Alpha ATR-FTIR spectrophotometer (Germany). The sample (25 mg) was applied directly to the superficial mass detector of the instrument without the need to prepare KBr disk. The band positions in the FTIR spectrum are shown as Wavenumber (υ, cm^{-1}), whereas the band intensities are expressed as Transmittance %.

2.5. Ultraviolet (UV)

The UV/Visible spectra were recorded by using UVD-2950(LABOMED, USA). The sample was prepared by mixing (10 µM) of the investigated product with 10 ml EtOH, and 2 ml of the resulted solution was placed in the instrument's cell for the investigation. The wavelength of maximum absorption (λmax) was recorded in nm for the synthetic products.

2.6. Nuclear magnetic resonance (NMR)

The proton-nuclear magnetic resonance (^{1}H-NMR) and carbon-nuclear magnetic resonance (^{13}C-NMR) spectra of the synthetic products were scanned on Bruker Analytische Messtechnik GmbH (400 MHz). To prepare the ^{1}H-NMR sample, 5mg of the investigated product was mixed in a small vial with 0.7 ml DMSO-d_6 for 10 min, and the resulted solution was transferred into the NMR tube by a glass pipette. For the ^{13}C-NMR sample preparation, 55mg of the investigated product was mixed in a small vial with 0.7 ml DMSO-d_6 for 30 min, and the resulted solution was transferred to the NMR tube by a glass pipette. The chemical shifts (δ) of these spectra were expressed in part per million (ppm) downfield to the internal standard tetramethylsilane (TMS). In an explanation of ^{1}H-NMR spectrum, the following terms were utilized to detect the spin-spin coupling: singlet (s), doublet (d), triplet (t) and multiplet (m).

2.7. Chemical synthesis

The designed steps adopted for the chemical synthesis of the target multifunctional coumarins (MC1-MC9) are depicted in Scheme 1.

2.7.1 Synthesis of 7-methoxycoumarin-4-acetic acid (MA1)

In a small conical flask immersed in a salt-ice bath, concentrated H_2SO_4 (10 ml) was added. As the acid temperature dropped to 0°C, citric acid (0.96 g, 5 mmol) was added in tiny portions over 2 hr. The frequency of addition was controlled by observing the temperature, which was maintained below 4°C. Subsequently, the reaction mixture was stirred at room temperature (RT) for 16 hr in a slow-motion style, poured on an ice-water mixture contained in a beaker, and filtered. The gray crystals of acetone dicarboxylic acid were washed with ethyl acetate (25 ml × 3), dried by suction, weighted, and directly used in the next step (Swamy *et al.*, 2015).

A solution of acetone dicarboxylic acid (1.46 g, 10 mmol) in 10 ml concentrated H_2SO_4 was stirred in a salt-ice bath for 30 min. The stirring was continued at RT until the evolution of gas was arrested. m-Guaiacol (1.08 ml, 10 mmol) was added to the above solution very slowly over 2 hr, and the reaction mixture was stirred in an ice-water bath. Then, the mixture was stirred at RT for two days, poured on an ice-water mixture, and filtered. The crude was dissolved in NaOH (50 ml, 1N), filtered, acidified, and the solid product was recrystallized from ethanol (Swamy *et al.*, 2015).

MA1: mp=184-186°C; λ_{max} (EtOH)=296 nm; % yield=49; R_f = 0.56; FTIR (v, stretching, cm^{-1}) 3095 (C-H, alkene), 3005 (O-H, COOH), 2884 (C-H, alkane), 1725 (C=O, ester), 1706 (C=O, COOH), 1692 (C=C, alkene), 1581 (C=O, aromatic), 1252 and 1071 (C-O-C, ether).

2.7.2 Synthesis of 7-methoxycoumarin-4-carbaldehyde (MA2)

A precooled mixture of **MA1** (1.17 g, 5 mmol) in 25 ml dry ether was added dropwise to a stirred solution of lithium tri-*tert*-butoxyaluminum hydride ($LiAlH(OtBu)_3$, 1.27 g, 5 mmol) in 75 ml dry ether at 0°C. As the addition ended, the reaction mixture was stirred for 1 hr in an ice-water bath. To this, HCl (1N) was added and the pH of the resulted mixture was justified at 5. The product

was extracted by ethyl acetate, washed with saturated NaCl solution, dried over sodium sulfate, and vaporized. The target product was recrystallized from CH_2Cl_2 (Fessler *et al.*, 2013).

MA2: mp=152-155°C; λ_{max} (EtOH)=289 nm; % yield=51; R_f = 0.61; FTIR (v, stretching, cm^{-1}) 3062 (C-H, alkene), 2912 (C-H, alkane), 2724 (C-H, aldehyde), 1722 (C=O, ester), 1700 (C=O, aldehyde), 1690 (C=C, alkene), 1580 (C=O, aromatic), 1248 and 1063 (C-O-C, ether).

2.7.3 Acid-catalyzed synthesis of 7-methoxy-4-but-2-enal derivatives (MA3-MA5)

A solution of acetaldehyde derivative (5 mmol) in 20 ml concentrated H_2SO_4 was stirred at RT for 30 min. To this, a solution of **MA2** (1.09 g, 5 mmol) in 30 ml ether was added dropwise over 15 min. The reaction mixture was refluxed for 24 hr, poured on an ice-water mixture, filtered, and washed with water several times. The crude product was recrystallized from a mixture of ether: ethyl acetate (Amarasekara and Ha, 2018).

MA3: mp=136-139°C; λ_{max} (EtOH)=311 nm; % yield=63; R_f = 0.63; FTIR (v, stretching, cm^{-1}) 3043 (C-H, alkene), 2916 (C-H, alkane), 2710 (C-H, aldehyde), 1727 (C=O, ester), 1691 (C=O, aldehyde), 1688, 1627 (C=C, alkene), 1582 (C=O, aromatic), 1247 and 1057 (C-O-C, ether).

MA4: mp=142-145°C; λ_{max} (EtOH)=310 nm; % yield=57; R_f = 0.66; FTIR (v, stretching, cm^{-1}) 3046 (C-H, alkene), 2919 (C-H, alkane), 2709 (C-H, aldehyde), 1724 (C=O, ester), 1694 (C=O, aldehyde), 1690, 1625 (C=C, alkene), 1582 (C=O, aromatic), 1242 and 1057 (C-O-C, ether).

MA5: mp=140-143°C; λ_{max} (EtOH)=321 nm; % yield=58; R_f = 0.67; FTIR (v, stretching, cm^{-1}) 3062 (C-H, alkene), 2953 (C-H, alkane), 2722 (C-H, aldehyde), 1720 (C=O, ester), 1686 (C=O, aldehyde), 1698, 1624 (C=C, alkene), 1589 (C=O, aromatic), 1240 and 1065 (C-O-C, ether).

2.7.4 Synthesis of multifunctional coumarins (MC1-MC9)

A mixture of 7-methoxy-4-but-2-enal derivative (2 mmol) and toluidine derivative (2 mmol) in 25 ml benzene was refluxed for 30 min. As its temperature dropped to RT, the reaction mixture was filtered and the solid was washed with cold benzene (10 ml × 3) then with n-hexane (10 ml × 3). The mother filtrate combined with the washings was concentrated under vacuum and the product was recrystallized from ethyl acetate (Sanap and Samant, 2015).

7-methoxy-4-((2*E*)-4-(o-tolylimino)but-2-en-1-yl)-2*H*-chromen-2-one (**MC1**): mp=150-153°C; λ_{max} (EtOH)=346 nm; % yield=81; R_f = 0.74; FTIR (v, stretching, cm^{-1}): 3049 (C-H, alkene), 2910 (C-H, alkane), 1727 (C=O, ester), 1682, 1626 (C=C, alkene), 1639 (N=C), 1582 (C=O, aromatic), 1243 and 1052 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.24 (1H, d, H-5), 8.12 (1H, d, H-14), 8.02 (1H, t, H-19), 7.60 (1H, d, H-17), 7.32 (1H, d, H-20), 7.06 (1H, s, H-8), 6.91 (1H, d, H-7), 6.83 (1H, t, H-18), 6.50 (1H, s, H-3), 6.12 (1H, t, *J*= 15 Hz, H-12), 5.65 (1H, t, *J*= 15 Hz, H-13), 4.24 (3H, s, OCH_3), 3.02 (2H, d, H-11), 2.85 (3H, s, H-21) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 165.8 (CH, C-14), 162.4 (C, C-2), 158.1 (C, C-7), 156.1 (C, C-4), 155.0 (C, C-9), 149.6 (C, C-15), 144.9 (C, C-4), 139.5 (CH, C-12), 132.9 (CH, C-17), 130.1 (C, C-16), 128.3 (CH, C-18), 127.0 (CH, C-19), 124.8 (CH, C-5), 124.0 (CH, C-13), 121.2 (CH, C-20), 115.2 (C, C-10), 113.5 (CH, C-3), 111.8 (CH, C-6), 103.8 (CH, C8), 57.7 (CH_3, OCH_3-C-7), 44.6 (CH_2, C-11), 22.7 (CH_3, C-21) ppm.

7-methoxy-4-((2*E*)-4-(m-tolylimino)but-2-en-1-yl)-2*H*-chromen-2-one (**MC2**): mp=159-162°C; λ_{max} (EtOH)=345 nm; % yield=85; R_f = 0.76; FTIR (v, stretching, cm^{-1}): 3044 (C-H, alkene), 2916 (C-H, alkane), 1724 (C=O, ester), 1681, 1628 (C=C, alkene), 1642 (N=C), 1588 (C=O, aromatic), 1249 and 1060 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.22 (1H, d, H-5), 8.15 (1H, d, H-14), 8.01 (1H, t, H-19), 7.36 (1H, s, H-16), 7.25 (1H, d, H-18), 7.17 (1H, d, H-20), 7.05 (1H, s, H-8), 6.90 (1H, d, H-7), 6.53 (1H, s, H-3), 6.11 (1H, t, *J*= 15 Hz, H-12), 5.67 (1H, t, *J*= 15 Hz, H-13), 4.27 (3H, s, OCH_3), 3.03 (2H, d, H-11), 2.86 (3H, s, H-21) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 165.5 (CH, C-14), 162.7 (C, C-2), 158.2 (C, C-7), 156.6 (C, C-4), 155.1 (C, C-9), 151.6 (C, C-15), 145.0 (C, C-4), 142.5 (C, C-17), 140.1 (CH, C-12), 129.9 (CH, C-19), 125.1 (CH, C-18), 124.9 (CH, C-5), 123.6 (CH, C-13), 122.4 (CH, C-16), 119.6 (CH, C-20), 115.4 (C, C-10), 113.1 (CH, C-3), 112.0 (CH, C-6), 104.1 (CH, C8), 57.8 (CH_3, OCH_3-C-7), 44.5 (CH_2, C-11), 25.2 (CH_3, C-21) ppm.

7-methoxy-4-((2*E*)-4-(p-tolylimino)but-2-en-1-yl)-2*H*-chromen-2-one (**MC3**): mp=144-147°C; λ_{max} (EtOH)=342 nm; % yield=79; R_f = 0.76; FTIR (v, stretching, cm^{-1}): 3052 (C-H, alkene), 2892 (C-H, alkane), 1725 (C=O, ester), 1680, 1625 (C=C, alkene), 1640 (N=C), 1583 (C=O, aromatic), 1242 and 1058 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.27 (1H, d, H-5), 8.14 (1H, d, H-14), 8.08 (1H, d, H-16), 7.84 (1H, d, H-19), 7.78 (1H, d, H-20), 7.08 (1H, s, H-8), 6.92 (1H, d, H-17), 6.87 (1H, d, H-7), 6.47 (1H, s, H-3), 6.06 (1H, t, *J*= 15 Hz, H-12), 5.66 (1H, t, *J*= 15 Hz, H-13), 4.24 (3H,

s, OCH_3), 3.08 (2H, d, H-11), 2.82 (3H, s, H-21) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 165.2 (CH, C-14), 162.9 (C, C-2), 159.0 (C, C-7), 155.9 (C, C-4), 153.7 (C, C-9), 150.2 (C, C-15), 145.2 (C, C-4), 141.5 (C, C-18), 141.3 (CH, C-12), 131.1 (CH, C-16), 127.4 (CH, C-19), 125.4 (CH, C-5), 124.1 (CH, C-20), 123.8 (CH, C-13), 123.5 (CH, C-17), 115.5 (C, C-10), 113.1 (CH, C-3), 112.1 (CH, C-6), 105.2 (CH, C8), 57.7 (CH_3, OCH_3-C-7), 44.4 (CH_2, C-11), 22.1 (CH_3, C-21) ppm.

7-methoxy-4-((2*E*)-3-methyl-4-(o-tolylimino)but-2-en-1-yl)-2*H*-chromen-2-one (**MC4**): mp=154-156°C; λ_{max} (EtOH)=346 nm; % yield=82; R_f= 0.79; FTIR (v, stretching, cm^{-1}): 3051 (C-H, alkene), 2929 (C-H, alkane), 1722 (C=O, ester), 1680, 1621 (C=C, alkene), 1632 (N=C), 1589 (C=O, aromatic), 1252 and 1060 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.20 (1H, d, H-5), 8.14 (1H, s, H-14), 8.00 (1H, t, H-19), 7.66 (1H, d, H-17), 7.37 (1H, d, H-20), 7.03 (1H, s, H-8), 6.96 (1H, d, H-7), 6.75 (1H, t, H-18), 6.47 (1H, s, H-3), 5.90 (1H, t, H-12), 4.26 (3H, s, OCH_3), 3.10 (2H, d, H-11), 2.86 (3H, s, H-21), 2.37 (3H, s, CH_3-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 165.2 (CH, C-14), 162.7 (C, C-2), 158.2 (C, C-7), 156.1 (C, C-4), 155.6 (C, C-9), 150.1 (C, C-15), 145.2 (C, C-4), 141.2 (CH, C-12), 133.4 (CH, C-17), 130.9 (C, C-16), 128.8 (CH, C-18), 126.7 (CH, C-19), 124.2 (CH, C-5), 121.5 (C, C-13), 118.4 (CH, C-20), 115.2 (C, C-10), 113.5 (CH, C-3), 110.1 (CH, C-6), 103.3 (CH, C8), 57.1 (CH_3, OCH_3-C-7), 38.1 (CH_2, C-11), 22.8 (CH_3, C-21), 18.6 (CH_3, CH_3-C-13) ppm.

7-methoxy-4-((2*E*)-3-methyl-4-(m-tolylimino)but-2-en-1-yl)-2*H*-chromen-2-one (**MC5**): mp=167-170°C; λ_{max} (EtOH)=342 nm; % yield=78; R_f= 0.79; FTIR (v, stretching, cm^{-1}): 3064 (C-H, alkene), 2925 (C-H, alkane), 1727 (C=O, ester), 1680, 1628 (C=C, alkene), 1649 (N=C), 1583 (C=O, aromatic), 1255 and 1043 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.30 (1H, d, H-5), 8.12 (1H, s, H-14), 8.00 (1H, t, H-19), 7.32 (1H, s, H-16), 7.26 (1H, d, H-18), 7.18 (1H, d, H-20), 7.07 (1H, s, H-8), 6.88 (1H, d, H-7), 6.50 (1H, s, H-3), 5.83 (1H, t, H-12), 4.28 (3H, s, OCH_3), 3.09 (2H, d, H-11), 2.88 (3H, s, H-21), 2.39 (3H, s, CH_3-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 166.4 (CH, C-14), 161.3 (C, C-2), 159.0 (C, C-7), 157.4 (C, C-4), 155.0 (C, C-9), 152.7 (C, C-15), 146.2 (C, C-4), 142.3 (C, C-17), 141.7 (CH, C-12), 130.2 (CH, C-19), 125.3 (CH, C-18), 124.6 (CH, C-5), 122.4 (CH, C-16), 121.4 (C, C-13), 119.1 (CH, C-20), 115.1 (C, C-10), 113.8 (CH, C-3), 112.2 (CH, C-6), 103.4 (CH, C8), 58.2 (CH_3, OCH_3-C-7), 38.8 (CH_2, C-11), 25.0 (CH_3, C-21), 18.4 (CH_3, CH_3-C-13) ppm.

7-methoxy-4-((2*E*)-3-methyl-4-(p-tolylimino)but-2-en-1-yl)-2*H*-chromen-2-one (**MC6**): mp=153-155°C; λ_{max} (EtOH)=340 nm; % yield=76; R_f= 0.79; FTIR (v, stretching, cm^{-1}): 3062 (C-H, alkene), 2902 (C-H, alkane), 1727 (C=O, ester), 1677, 1623 (C=C, alkene), 1635 (N=C), 1585 (C=O, aromatic), 1249 and 1055 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.22 (1H, d, H-5), 8.12 (1H, s, H-14), 8.02 (1H, d, H-16), 7.89 (1H, d, H-19), 7.76 (1H, d, H-20), 7.12 (1H, s, H-8), 6.90 (1H, d, H-17), 6.76 (1H, d, H-7), 6.44 (1H, s, H-3), 5.77 (1H, t, H-12), 4.28 (3H, s, OCH_3), 3.13 (2H, d, H-11), 2.86 (3H, s, H-21), 2.42 (3H, s, CH_3-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 167.3 (CH, C-14), 164.2 (C, C-2), 157.1 (C, C-7), 154.2 (C, C-4), 153.3 (C, C-9), 150.2 (C, C-15), 145.7 (C, C-4), 141.8 (C, C-18), 141.1 (CH, C-12), 131.8 (CH, C-16), 127.5 (CH, C-19), 125.5 (CH, C-5), 124.3 (CH, C-20), 123.8 (CH, C-17), 121.4 (CH, C-13), 116.2 (C, C-10), 114.3 (CH, C-3), 110.3 (CH, C-6), 105.5 (CH, C8), 53.4 (CH_3, OCH_3-C-7), 37.6 (CH_2, C-11), 22.8 (CH_3, C-21), 19.1 (CH_3, CH_3-C-13) ppm.

7-methoxy-4-((2*E*)-3-((o-tolylimino)methyl)pent-2-en-1-yl)-2*H*-chromen-2-one (**MC7**): mp=160-163°C; λ_{max} (EtOH)=345 nm; % yield=76; R_f= 0.80; FTIR (v, stretching, cm^{-1}): 3066 (C-H, alkene), 2912 (C-H, alkane), 1727 (C=O, ester), 1678, 1620 (C=C, alkene), 1637 (N=C), 1588 (C=O, aromatic), 1250 and 1052 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.21 (1H, d, H-5), 8.11 (1H, s, H-14), 8.00 (1H, t, H-19), 7.68 (1H, d, H-17), 7.36 (1H, d, H-20), 7.10 (1H, s, H-8), 6.92 (1H, d, H-7), 6.71 (1H, t, H-18), 6.45 (1H, s, H-3), 5.95 (1H, t, H-12), 4.22 (3H, s, OCH_3), 3.13 (2H, d, H-11), 2.84 (3H, s, H-21), 2.60 (2H, q, *J*= 9 Hz, $C\underline{H}_2CH_3$-13), 1.86 (3H, t, *J*= 9 Hz, $CH_2C\underline{H}_3$-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 165.2 (CH, C-14), 162.7 (C, C-2), 158.2 (C, C-7), 156.1 (C, C-4), 155.6 (C, C-9), 150.1 (C, C-15), 145.2 (C, C-4), 140.4 (CH, C-12), 133.4 (CH, C-17), 131.5 (C, C-13), 130.9 (C, C-16), 128.8 (CH, C-18), 126.7 (CH, C-19), 124.2 (CH, C-5), 118.4 (CH, C-20), 115.2 (C, C-10), 113.5 (CH, C-3), 110.1 (CH, C-6), 103.3 (CH, C8), 57.1 (CH_3, OCH_3-C-7), 38.8 (CH_2, C-11), 22.8 (CH_3, C-21), 20.4 (CH_2, $\underline{C}H_2CH_3$-C-13), 16.8 (CH_3, $CH_2\underline{C}H_3$-C-13) ppm.

7-methoxy-4-((2*E*)-3-((m-tolylimino)methyl)pent-2-en-1-yl)-2H-chromen-2-one (**MC8**): mp=173-176°C; λ_{max} (EtOH)=347 nm; % yield=71; R_f= 0.80; FTIR (v, stretching, cm^{-1}): 3073 (C-H, alkene), 2908 (C-H, alkane), 1724 (C=O, ester), 1675, 1630 (C=C, alkene), 1640 (N=C), 1584 (C=O, aromatic), 1250 and 1042 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.26 (1H, d, H-5), 8.15 (1H, s, H-14), 7.89 (1H, t, H-19), 7.41 (1H, s, H-

16), 7.33 (1H, d, H-18), 7.19 (1H, d, H-20), 7.02 (1H, s, H-8), 6.87 (1H, d, H-7), 6.46 (1H, s, H-3), 5.78 (1H, t, H-12), 4.30 (3H, s, OCH_3), 3.06 (2H, d, H-11), 2.90 (3H, s, H-21), 2.67 (2H, q, *J*= 9 Hz, $C\underline{H}_2CH_3$-13), 1.82 (3H, t, *J*= 9 Hz, $CH_2C\underline{H}_3$-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 164.2 (CH, C-14), 161.6 (C, C-2), 158.1 (C, C-7), 156.2 (C, C-4), 154.4 (C, C-9), 152.3 (C, C-15), 146.2 (C, C-4), 142.6 (C, C-17), 140.1 (CH, C-12), 130.9 (C, C-13), 128.1 (CH, C-19), 125.8 (CH, C-18), 124.0 (CH, C-5), 122.4 (CH, C-16), 119.5 (CH, C-20), 115.2 (C, C-10), 113.8 (CH, C-3), 110.9 (CH, C-6), 103.4 (CH, C8), 58.5 (CH_3, OCH_3-C-7), 39.1 (CH_2, C-11), 24.6 (CH_3, C-21), 21.1 (CH_2, $\underline{C}H_2CH_3$-C-13), 17.2 (CH_3, $CH_2\underline{C}H_3$-C-13) ppm.

7-methoxy-4-((2*E*)-3-((p-tolylimino)methyl)pent-2-en-1-yl)-2H-chromen-2-one (**MC9**): mp=168-171°C; λ_{max} (EtOH)=340 nm; % yield=73; R_f= 0.81; FTIR (v, stretching, cm^{-1}): 3055 (C-H, alkene), 2911 (C-H, alkane), 1726 (C=O, ester), 1680, 1620 (C=C, alkene), 1632 (N=C), 1586 (C=O, aromatic), 1243 and 1050 (C-O-C, ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 8.20 (1H, d, H-5), 8.11 (1H, s, H-14), 8.01 (1H, d, H-16), 7.92 (1H, d, H-19), 7.72 (1H, d, H-20), 7.10 (1H, s, H-8), 6.94 (1H, d, H-17), 6.72 (1H, d, H-7), 6.46 (1H, s, H-3), 5.74 (1H, t, H-12), 4.31 (3H, s, OCH_3), 3.18 (2H, d, H-11), 2.84 (3H, s, H-21), 2.64 (2H, q, *J*= 9 Hz, $C\underline{H}_2CH_3$-13), 1.80 (3H, t, *J*= 9 Hz, $CH_2C\underline{H}_3$-13) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 165.1 (CH, C-14), 162.8 (C, C-2), 155.9 (C, C-7), 154.7 (C, C-4), 153.2 (C, C-9), 149.1 (C, C-15), 145.7 (C, C-4), 141.9 (CH, C-12), 139.2 (C, C-18), 133.2 (CH, C-16), 130.9 (CH, C-13), 128.3 (CH, C-19), 125.3 (CH, C-5), 124.7 (CH, C-20), 122.3 (CH, C-17), 116.9 (C, C-10), 114.0 (CH, C-3), 109.6 (CH, C-6), 103.9 (CH, C8), 53.5 (CH_3, OCH_3-C-7), 37.9 (CH_2, C-11), 24.2 (CH_3, C-21), 20.5 (CH_2, $\underline{C}H_2CH_3$-C-13), 17.8 (CH_3, $CH_2\underline{C}H_3$-C-13) ppm.

2.8. Introductory antitumor activity

In a sheet of 96 holes, the cells (10.000) of the chosen cancer line were dispersed in each single hole and subsequently exposed in the next 24 hr to the screened products at a defined concentration. The applied concentrations that ranged from 200 µg/ml to 6.25 µg/ml were prepared from a stock DMSO solution (1 mM) in a double-dilution manner. The MTT test was initiated in the next 72 hr of exposure by detaching the medium, applying 26 µl of the MTT reagent (3.23 mM), and posteriorly incubating the exposed cells for 90 min at 37°C. The cell viability was estimated via a multi-mode microplate reader adjusted at 492 nm by following the absorbances of the exposed hole (A_E) and unexposed hole (A_U). The cytotoxic evaluation of the screened products was verified by calculating the growth inhibition (GI) percentage via the following mathematical rule: GI % = $(A_U - A_E)/A_U \times 100$ (Borges *et al.*, 2005; Altemimi *et al.*, 2017; Nejres *et al.*, 2020).

3. RESULTS AND DISCUSSION:

3.1. Chemical approach

The synthetic plan for preparing the target products was depicted in Scheme 1. This plan commenced by utilizing the strong acidic feature of H_2SO_4 to transform citric acid to acetone dicarboxylic acid, which was condensed via a Pechmann reaction with m-guaiacol affording 7-methoxycoumarin-4-acetic acid (**MA1**) (Hari Krishna and Thriveni, 2016; Bouasla *et al.*, 2017; Jung *et al.*, 2018; Melita Lonˇcari´c, Dajana Gašo-Sokaˇc, 2020). The carboxylic acid moiety of the aforementioned product was reduced to aldehyde group by using a mild reducing agent, $LiAlH(OtBu)_3$, which was selected to avoid the cleavage of the lactone ring (Liu *et al.*, 2017) yielding **MA2** product. In the next step, the aldol condensation reaction was designed to involve the nucleophilic attack of the enol form of **MA2** to the protonated aldehyde moiety of the employed acetaldehyde derivatives affording **MA3**-**MA5** products (Amarasekara and Ha, 2018). The aldehyde groups of these products were coupled with the primary amine groups of the utilized toluidine derivatives to generate Schiff-base containing products termed multifunctional coumarin derivatives (**MC1**-**MC9**)(Purkait *et al.*, 2016; Al Zoubi *et al.*, 2018; van Schijndel *et al.*, 2019). The design of the synthetic plan, as well as the characterization data, confirmed that the chemical structures represented in Figure 1 could be attributed to the target synthetic products.

3.2 Introductory antitumor activity

The cytotoxic estimation of the synthesized multifunctional coumarins (**MC1**-**MC9**) was inspected with MTT dye to determine the cell viability. The cancer lines utilized in this estimation included HeLa (cervix), SKG (esophageal), MCF-7 (breast), and AMN3 (murine mammary adenocarcinoma). In this study, six concentrations of the individual product were prepared via serial double-dilution and employed to calculate the IC_{50} values. Also, the solvent DMSO and the standard cytotoxic agent 5-fluorouracil were employed as negative and positive leading reagents respectively (Saha *et al.*, 2017; Haq *et al.*, 2019; Stringlis *et al.*, 2019).

The outcomes illustrated in Table 1 and explained in Figure 2 reflect several interesting considerations. Firstly, IC_{50} values of the synthesized multifunctional coumarins are generally higher than that of the positive standard. Secondly, the position of R' group has a notable effect on the cytotoxicity; the order of increasing effect is 4-CH_3 > 2-CH_3 > 3-CH_3. From this order, it is concluded that the cytotoxicity may be enhanced by substituting the aromatic ring with an electron-donating group in such a manner to increase the inductive effect toward the nitrogen portion of the Schiff-base (Purkait *et al.*, 2016; Dos Santos *et al.*, 2017; Teran *et al.*, 2019).

Thirdly, the steric factor exerted by the group substituted on the carbon next to the carbon bound to the Schiff-base nitrogen has an effect on the cytotoxicity (Purkait *et al.*, 2016). This can be clarified by observing the decline in the antitumor activity of the synthesized products as this substituted group becomes bulkier. In this context, compounds **MC1**, **MC2**, and **MC3** have a better antitumor activity versus the test cell lines than that of their corresponding compounds **MC4**, **MC5**, and **MC6** which in turn have a better activity than that of compounds **MC7**, **MC8**, and **MC9**.

Fourthly, the steric factor described in the third point exerts a higher impact on cytotoxicity than that of the inductive effect described in the second point (Fattuoni *et al.*, 2020). Fifthly, similar antitumor tendency of the synthesized multifunctional coumarins was predestined versus the test cell lines, with the best activity being against MCF-7 cells and the least effect being versus AMN3 cells.

Finally, it can be assumed that the nitrogen portion of the Schiff-base may play a significant role in the cytotoxicity of the synthesized coumarins by participating in the product-target interactions as a hydrogen-bond acceptor (Chow *et al.*, 2014).

4. CONCLUSIONS:

Based on the results acquired from the antitumor activity versus four prevalent human tumor lines, compound **MC3** showed the best activity. From that, it is concluded the nitrogen fraction of the Schiff-base linkage may play a significant role as a hydrogen-bond acceptor in the antitumor activity of the synthesized coumarins. This role may rely on the steric factor on the carbon next to that attached to nitrogen and on the inductive factor exerted by the moiety linked directly to this nitrogen. The position of R' group has a significant effect on the cytotoxicity of the synthesized products; the order of increasing effect is 4-CH_3 > 2-CH_3 > 3-CH_3. From this order, it is concluded that the cytotoxicity may be enhanced by substituting the aromatic ring with an electron-donating group in such a manner to increase the inductive effect toward the nitrogen portion of the Schiff-base. Also, the steric factor described above exerts a higher impact on cytotoxicity than that of the inductive effect. Accordingly, these coumarins may represent a novel template for the evolution of new antitumor agents.

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6. CONFLICT OF INTEREST:

There are no conflicts of interest.

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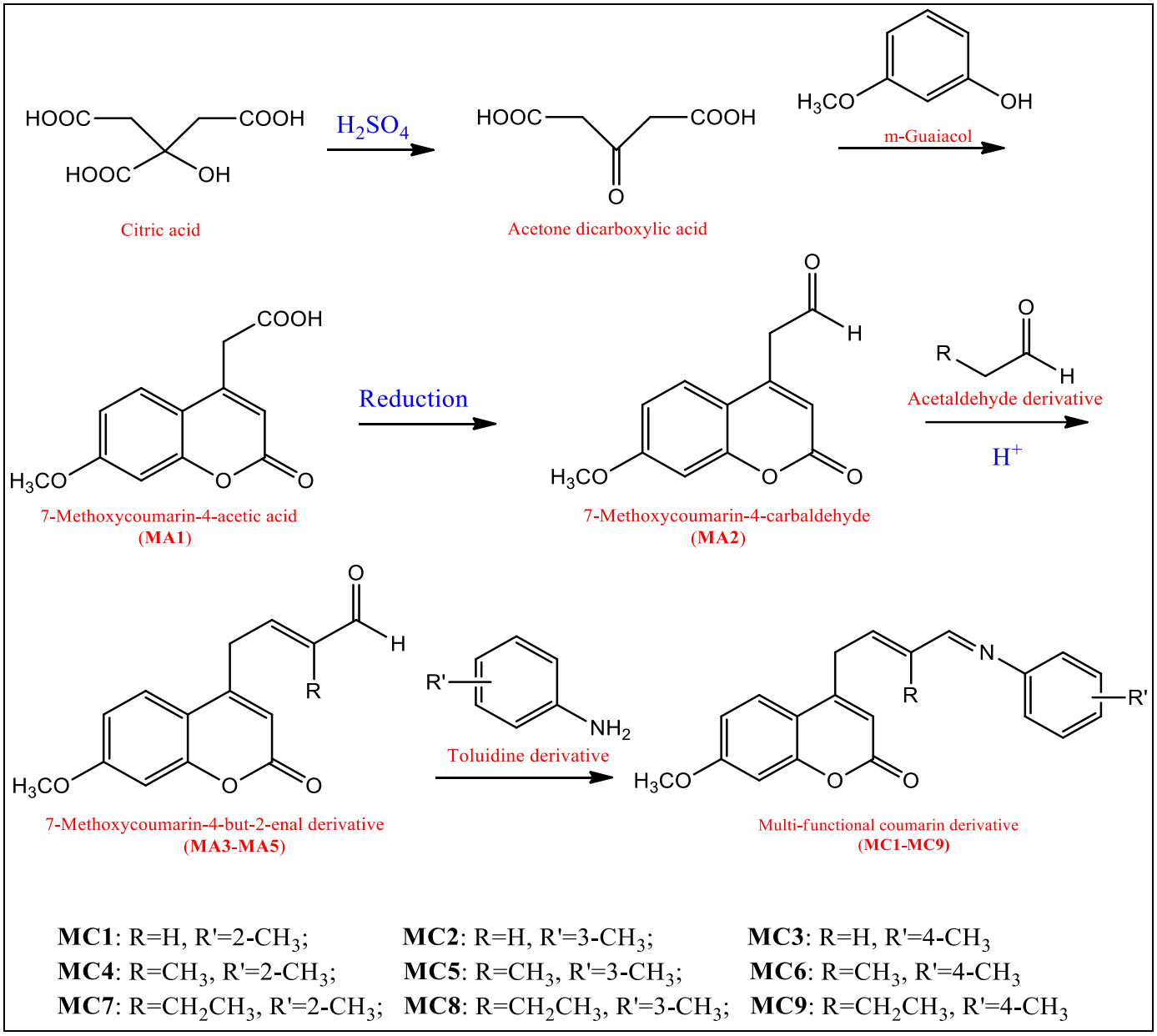

Scheme 1. *The designed steps adopted for the chemical synthesis of the target multifunctional coumarins (MC1-MC9).*

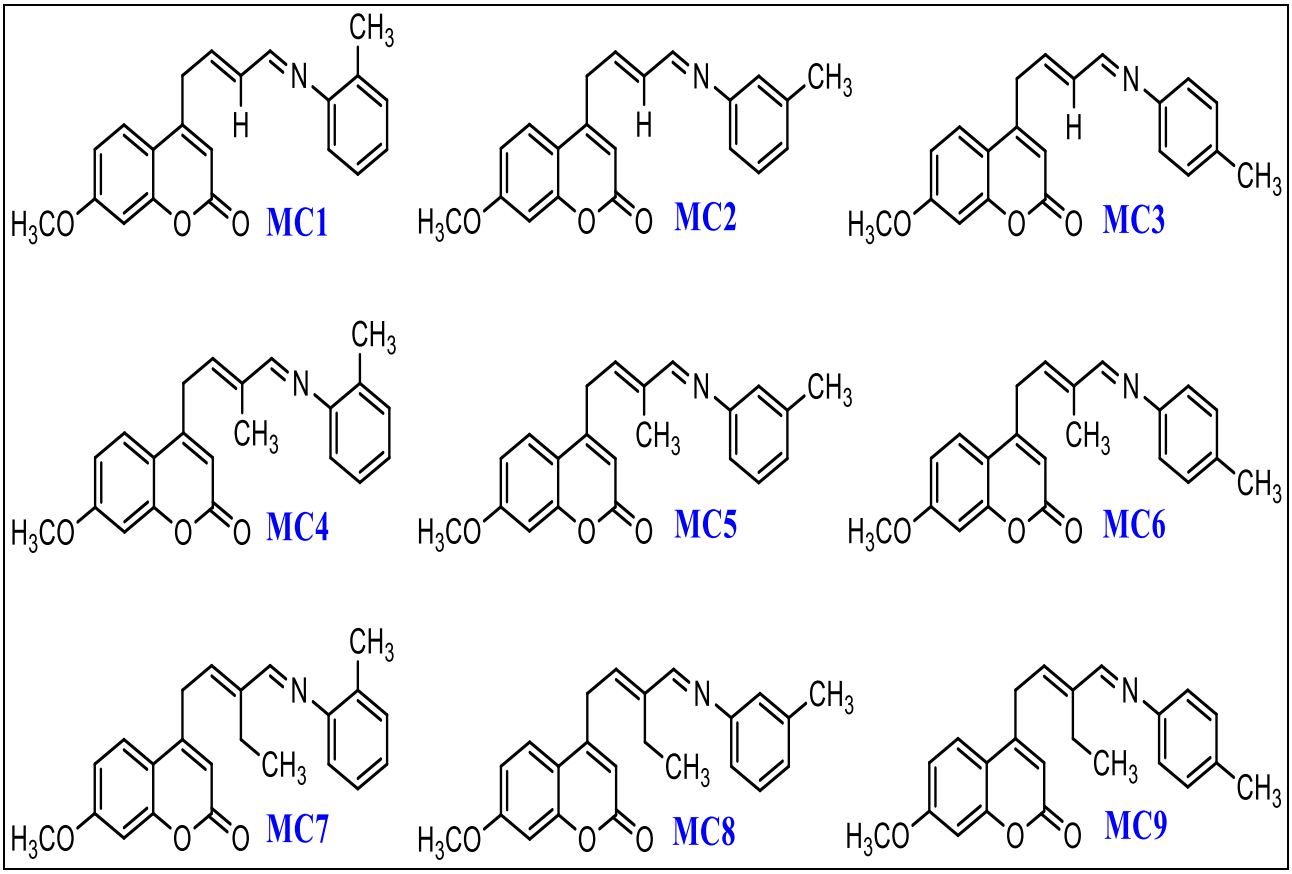

Figure 1. *Chemical structures of the target multifunctional coumarins (MC1-MC9).*

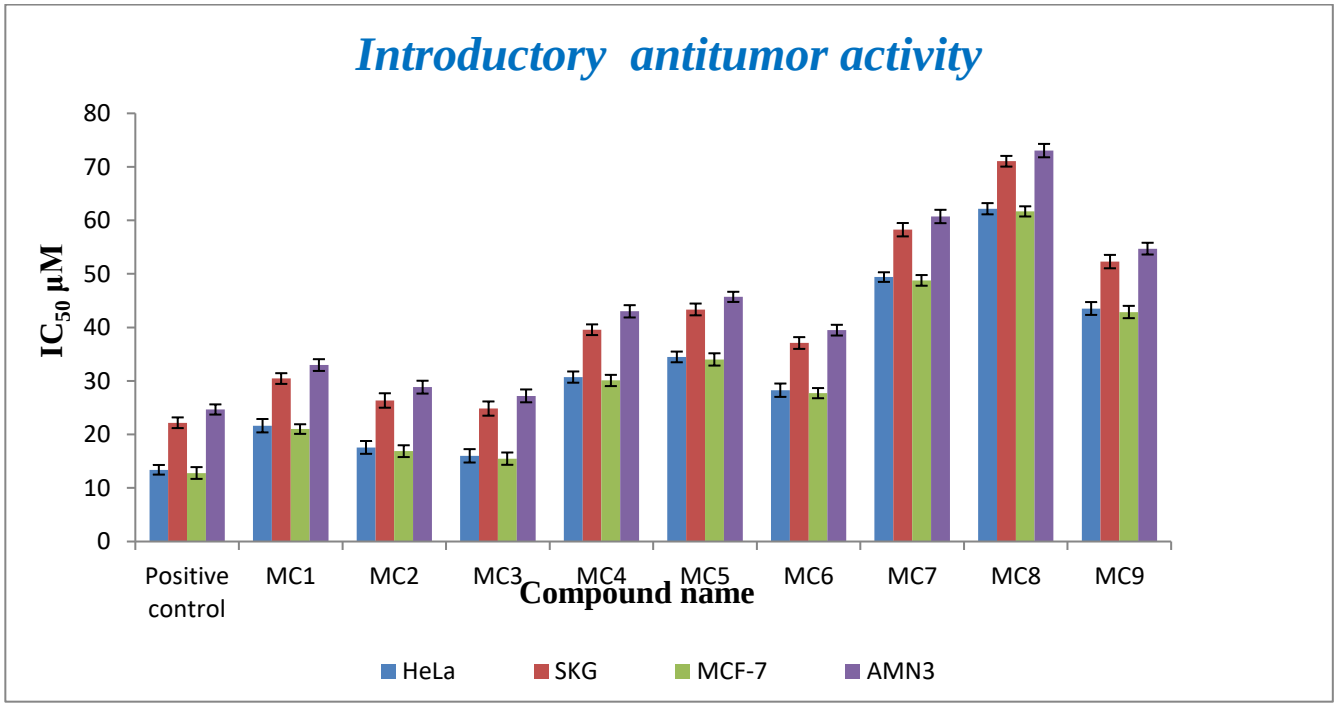

Figure 2. *Graph representing the IC_{50} values of the synthesized multifunctional coumarins and the positive control versus the employed tumor cell lines.*

Table 1. *Outcomes assumed from assaying the in vitro antitumor activity of the positive control and synthesized multifunctional coumarins utilizing MTT test.*

Compound Name	Introductory antitumor activity IC_{50} ± SD			
	HeLa	**SKG**	**MCF-7**	**AMN3**
Positive control*	13.40 ± 0.90	22.19 ± 1.00	12.80 ± 1.10	24.67 ± 0.95
MC1	21.64 ± 1.25	30.45 ± 1.00	21.01 ± 0.90	32.97 ± 1.10
MC2	17.58 ± 1.20	26.35 ± 1.35	16.88 ± 1.10	28.84 ± 1.20
MC3	16.01 ± 1.25	24.84 ± 1.33	15.48 ± 1.15	27.21 ± 1.20
MC4	30.72 ± 1.05	39.57 ± 1.00	30.09 ± 1.05	43.01 ± 1.15
MC5	34.49 ± 1.00	43.36 ± 1.10	34.02 ± 1.15	45.71 ± 0.95
MC6	28.27 ± 1.25	37.09 ± 1.10	27.72 ± 0.95	39.49 ± 1.00
MC7	49.41 ± 0.90	58.26 ± 1.25	48.79 ± 1.00	60.73 ± 1.25
MC8	62.18 ± 1.05	71.05 ± 1.00	61.68 ± 0.95	73.04 ± 1.25
MC9	43.54 ± 1.20	52.30 ± 1.25	42.88 ± 1.15	54.72 ± 1.10

Notes: * The positive control is 5-fluorouracil. IC_{50} are expressed in μM. SD is calculated from three independent experiments.