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Synthesis and Characterization of Some New Coumarin Derivatives as Probable Breast Anticancer MCF-7 Drugs

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Abstract: This study aimed to synthesize quinolinone derivatives and investigate their cytotoxic activity. The compound 1-azacoumarin-3-carboxylic acid (2-oxo-1H-quinoline-3-carboxylic acid) was obtained via the cyclocondensation of 2-hydroxybenzaldehyde with diethyl malonate in base catalyst to give ethyl coumarin-3-carboxylate, followed by the ammonolysis of ester (ethyl coumarin-3-carboxylate) with ammonia in the presence of anhydrous potassium carbonate. Treatment of 2-oxo-1H-quinoline-3-carboxylic acid with acetic anhydride, cinnamaldehyde, cinnamic acid and methyl 5-phenyl-2-cyano-2,4-pentadienoate under different conditions led to the formation of 1 (substituted) aza coumarin-3-carboxylic acids (1-*N*-(acetyl)-azacoumarin-3-carboxylic acid, 1-*N*-(2-Formyl-1-phenyl) vinyl-azacoumarin-3-carboxylic acids, 1-*N*-[2-(Hydroxy) carbonyl-1-(Phenyl) vinyl]-azacoumarin-3-carboxylic acid and 1-*N*-(4-Cyano-5-methoxy-5-oxo-1-Phenylpenta-1,3-diene-1-y)-azacoumarin-3-carboxylic 284 acid), respectively. The structures of synthesized 1-(substituted) azacoumarin-3-carboxylic acids were confirmed based on spectroscopic methods (IR and NMR), along with elemental analyses. Interestingly compound 6 demonstrated probable impacts as an anti-cancer drug against the MCF-7 cell line. The mechanism of action was assessed using a flow cytometric assay. The outcomes revealed that compound 6 could arrest the cell cycle at G2/M phase and pre-G1 apoptosis.

Keywords: anticancer drugs; quinolinone derivatives; MCF-7; cell cycle arrest

Citation: Gaber, A.; Alsanie, W.F.; Alhomrani, M.; Alamri, A.S.; El-Deen, I.M.; Refat, M.S. Synthesis and Characterization of Some New Coumarin Derivatives as Probable Breast Anticancer MCF-7 Drugs. *Crystals* **2021**, *11*, 565. https://doi.org/10.3390/cryst11050565

Academic Editors: Marian Valko and Younes Hanifehpour

Received: 24 March 2021
Accepted: 15 May 2021
Published: 19 May 2021

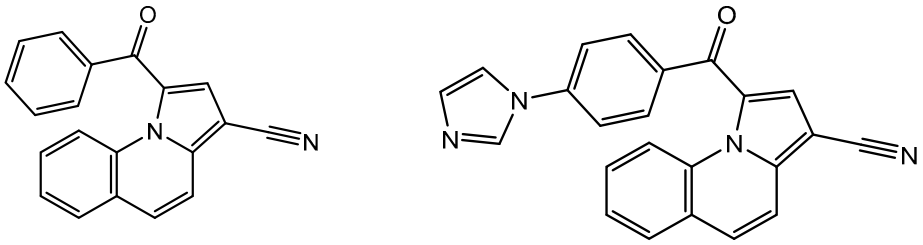
1. Introduction

Coumarins can be synthesized using a variety of methods, including the Perkin reaction, Knoevenagel condensation, Pechmann condensation, Wittig reaction, Baylis–Hillman reaction, Claisen rearrangement, and Vilsmeier–Haack and Suzuki cross-coupling reactions [1]. Many studies on the medicinal properties of coumarins have been published [2]. Coumarins have antimicrobial effects, including antibacterial [3] and antifungal [4] properties. Several coumarin derivatives have been shown to have antioxidant activity [5]. Some coumarins have been synthesized as acetylcholinesterase (AchE) inhibitors, which may be used to treat Alzheimer's disease [6]. Additionally, coumarins have a variety of biological properties, including anti-inflammatory [7], anti-HIV [8], anticancer [9], antituberculosis [10], anticoagulant [11], antiviral [12], and antihyperglycemic characteristics [13]. Coumarins have been proven to be efficient pharmacophores; therefore, demand for their synthesis is increasing. Many methods have been employed for their synthesis, each of which uses different starting materials and reaction conditions.

The green synthesis of coumarin derivatives in deep eutectic solvent (DES) by Knoevenagel condensation was reported by Keshavarzipour and Tavakol [14]. At 100 °C, DES was made by mixing choline chloride (ChCl) and zinc chloride. Easy or substituted salicylaldehydes (4hydroxy, 5bromo) and methylene compounds (dimethyl malonate, ethyl cyanoacetate, ethyl 3-oxo-3-phenylpropanoate) were used to make coumarin derivatives. The synthesis of coumarins by the Knoevenagel condensation of salicylaldehyde and diethyl malonate in EtOH was reported by Sulji and Pietruszka [15]. Piperidine and acetic acid were used as catalysts. In the method of continuous flow hydrogenation, chromanones were obtained from the synthesized coumarins.

Silveira Pinto and Souza used Knoevenagel condensation to synthesize various coumarins from active methylene compounds (diethylmalonate, Meldrum's acid, and ethylcyanoacetate) and salicylaldehydes [16]. Piperidine and glacial AcOH were added to the reaction, which was carried out in pure EtOH or H_2O. A model reaction of salicylaldehyde and diethyl malonate was carried out using a heating and ultrasonic process. In the case of ultrasound irradiation, the yield is higher and the reaction time is shorter, as compared to the reflux treatment (40 min compared with 7 h). Various coumarins were obtained using ultrasound irradiation at a frequency of 20 kHz with a power output of 90% and no pulsing.

From the published research, it has been reported that quinoline derivatives may have an anti-cancer effect through many diverse mechanisms, for example, cell cycle arrest in the G2 phase [17,18], inhibition of the formation of topoisomerase enzyme [18], inhibition of polymerization, and the formation of tubulin during the cell cycle [19]. The most well-known mechanism is inhibition of the tyrosine kinase enzyme [20–23], especially the enzyme known as the vascular epithelial growth factor receptor tyrosine kinase [24–27]. Kemnitzer et al. [28] found a new series of apoptosis inducers (Figure 1), the 1-benzoyl-3-cyanopyrrolo[1,2-*a*] quinolines (**I**), among which the compound 1-(4-(1-*h*-imidazol-1-yl)benzoyl)-3-cyanopyrrolo[1,2-*a*]quinoline (**II**) displayed high cytotoxic activity in T47D human breast cancer, HCT-116 human colon cancer cells, and SNU398 hepatocellular carcinoma cells.



(**A**) **I** (**B**) **II**

Figure 1. (**A**)Structures of 1-benzoyl-3-cyanopyrrolo[1,2-*a*] quinolines (**I**), and (**B**)1-(4-(1-*h*-imidazol-1-yl)benzoyl)-3-cyanopyrrolo[1,2-*a*]quinoline (**II**).

Coumarins are molecules that belong to a very special family [29]. The conjugated double-ring system gained the importance of the molecule in various fields of research [29]. Coumarin is used in contemporary industry, in many products; for example, cosmetics, perfumes, and as a food additive, in addition to its entry into the pharmaceutical industry, where it is used in the production of many synthetic pharmaceutical materials [30]. This last application is the main point of our research. There were many reports published in 2020 confirming the importance of coumarin use as an anti-cancer agent [31–35]. Additionally, the design of sulfamide 3-benzylcoumarin hybrids bearing an oxadiazole ring at position 7 has enabled the preparation of new multitarget mitogen-activated protein kinase inhibitors and nitric oxide donors, both of which can be used due to their antiproliferative properties [36].

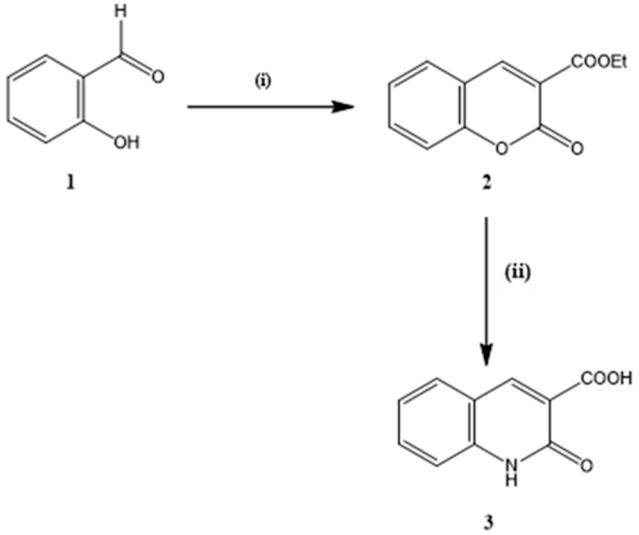
Encouraged by these observations and in continuation the work on the studies of coumarin as an anti-cancer drug, we report the synthesis of a new class of azacoumarin-3-carboxylic acid derivatives to determine the appropriate replacement/addition of different moieties leading to better inhibitors for cancer cells. These designed molecules have been synthesized and characterized by known methods with slight modifications wherever required. The presence of 1-*N*-(2-Formyl-1-phenyl) vinyl-azacoumarin-3-carboxylic acids (5), 1-*N*-[2-(Hydroxy) caybonyl-1-(Phenyl) vinyl]-azacoumarin-3-carboxylic acid (6), and 1-*N*-(4-Cyano-5-methoxy-5-oxo-1-Phenylpenta-1,3-diene-1-y)-azacoumarin-3-cayboxylic acid (9) compounds formed from the refluxed mixture of 2-oxo-1H-quinoline-3-carboxylic acid (3) (0.01 mole), and α,β-5-unsaturated carbonyl compounds (namely, cinnamaldehyde, cinnamic, acid and methyl 5-phenyl-2-cyano-2,4-pentadine oats (0.01 mole)) in 50 mL dimethylformamide for 4 h. The experimental data led us to design and synthesize the novel derivatives as probable MCF-7 anti-cancer moieties.

2. Results and Discussion

2.1. Chemistry

The synthetic pathway which leads to *N*-substituted azacoumarin-3-carboxylic acids (3–6 and 9) is presented in Schemes 1–3. The reaction sequence steps include: the cyclocondensation of 2-hydroxybenzaldehyde (1) with diethyl malonate in the presence of piperidine as a base catalyst to afford ethyl coumarin-3-carboxylate (2); and the ammonolysis of ester coumarin (2) with ammonia solution, in ethanol, in the presence of anhydrous potassium carbonate under reflux, which produced the corresponding 2-oxo-1*h*-quinoline-3-carboxylic acid (3) (Scheme 1).

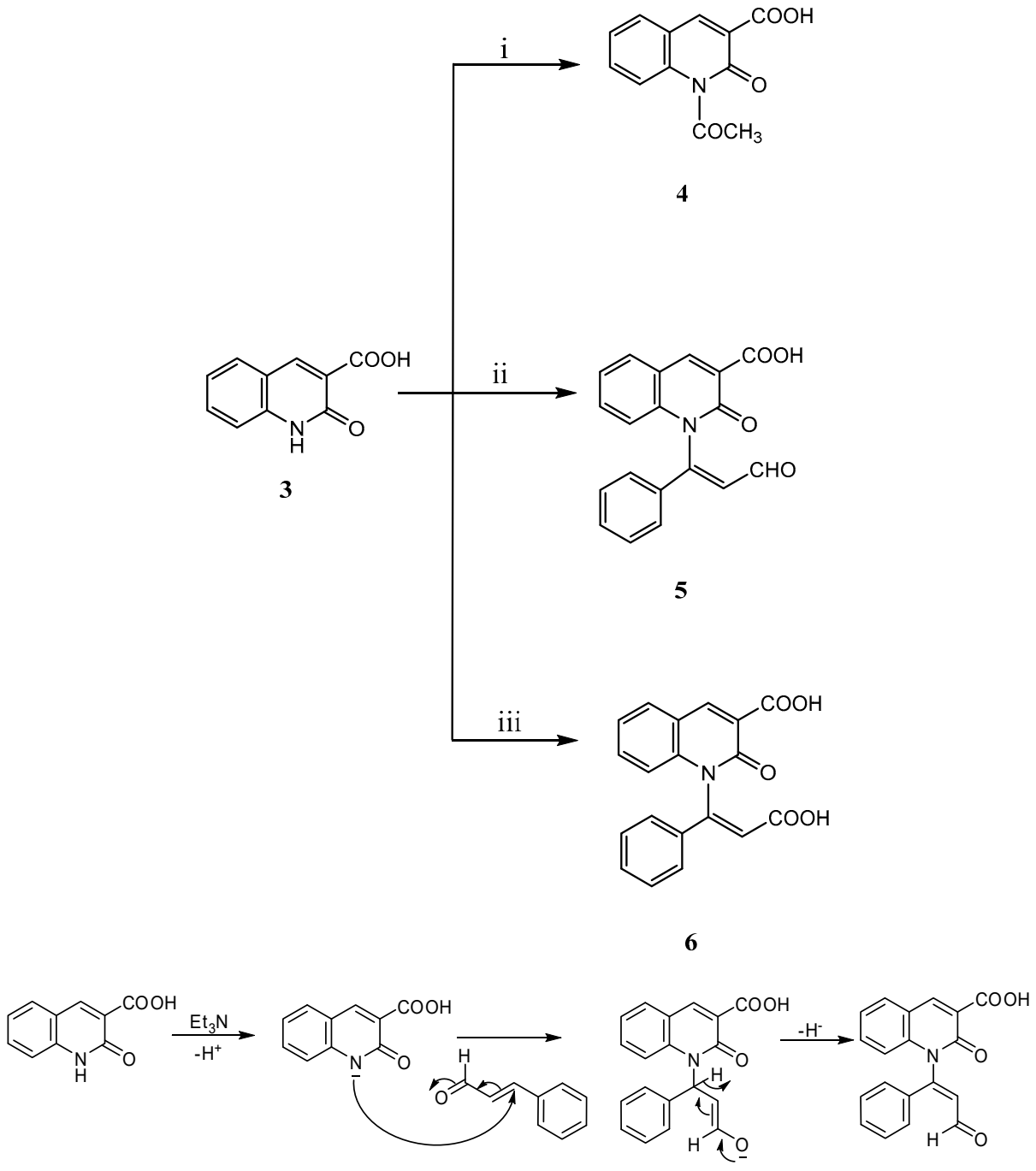
The structure of the achieved compounds was clarified by spectral data (Figures S1–S3 Supplementary Materials). The IR spectrum critical stretching bands fitting to OH, NH, C=O of acid and amide, C=C, and C–O groups were detected at the anticipated regions (Figure S1). The complete aromatic and pyridinone proton peaks in the H-NMR spectrum of compound **3** were in the estimated areas (Figure S2). The ^{13}C-NMR spectrum of compounds showed three carbon signals at δ = 163-00, 160.80 and 154.51 ppm, referring to the two carbonyl (C=O) and C–N groups (Figure S3). The signals present at δ = 148.28 ppm and signals within the region at 134–116.60 ppm are assigned to the C-4 carbon signal of 1-azacoumarin and aromatic carbons as well as C-3 of pyridinone rings.



Scheme 1. Synthesis of 1-azacoumarin-3-carboxylic acid (3). Reagents and reaction conditions: (i) diethyl malonate, piperidine/EtOH; (ii) ammonia solution (33%), K_2CO_3 /EtOH.

Additionally, the structure of compound **3** was verified chemically through its conversion into 1-*N*-(substituted)- azacoumarin-3- carboxylic acid (**4**) by boiling in acetic

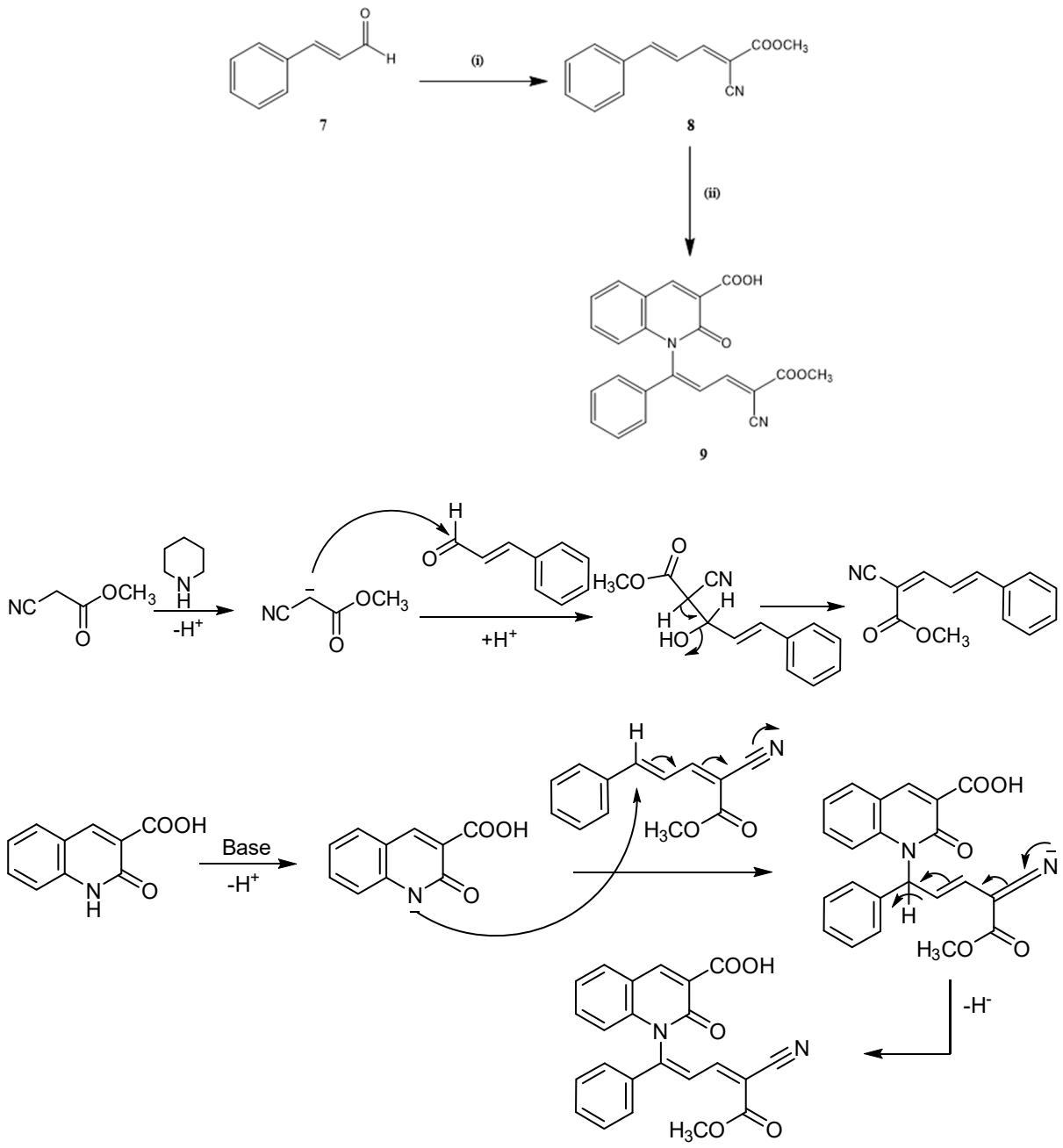
anhydride under reflux 1-*N*-(2-formyl-1-phenyl) vinylazacoumarin-3-carboxylic acids (**5**) and 1-*N*-[2-(hydroxy carbonyl)-1-(phenyl) vinylazacoumarine-3-carboxylic acid (**6**). Compounds **5** and **6** were synthesized by a condensation reaction of compound 3 with cinnamaldehyde and cinnamic acid in dimethyl formamide in the presence of triethylamine, followed by dehydrogenation under reflux (Scheme 2).



Scheme 2. Synthesis of 1-*N*-(substituted)-azacoumarin-3-Carboxylic acids (**4**–**6**). Reagents and reaction conditions: (i) acetic anhydride; (ii) cinnamaldehyde, Et_3N/DMF; (iii) cinnamic acids, Et_3N /DMF.

The data of ^{1}H-NMR spectrum of compound 4 displayed a new singlet signal at δ = 2.38, allocated to the protons of methyl function for the acetyl ($COCH_3$) group (Figure S4). The proton signals of 1-azacoumarin and hydroxyl (OH) groups were detected within the anticipated chemical shifts regions with the expected integral values (Figure S4). The

presence of new two carbon signals at δ = 190.59 and 21.27 ppm in the ^{13}C-NMR spectrum of compound indicated the presence of an acetyl (CH_3CO) group (Figure S5). These signals further supported the formation of compound 4. Additionally, the ^{13}C-NMR spectrum of compound 4 exhibited the same carbon signals as in compound 3, which gave a total of 12 carbon signals (Figure S5).



Scheme 3. Synthesis of 1-*N*-(4-cyano-5-methoxy-5-oxo-1-phenylpenta-b3-diene-l-y1)- azacoumarin-3-carboxylic acid (**9**). Reagents and reaction conditions: (i) methyl cyanoacetate, piperidine/MeOH; (ii) 1-*N*- azacoumarin-3-carboxylic acid, Et_3N/DMF.

The ^{1}H-NMR spectra of compounds **5** and **6** exhibited sharp singlet signals at δ = 9.83 and 12.46 ppm due to the protons of formyl (CHO) and carboxylic (COOH) groups (Figures S6 and S8). The H-4 protons of azacoumarin demonstrated a singlet signal at δ = 8.89 ppm in compounds **5** and **6**. Protons of the aromatic ring and olefinic were observed as multiple signals in the region at δ = 6.54–8.10 ppm. Additionally, the ^{13}C-

NMR spectra of compounds **5** and **6** displayed two new carbon signals at δ = 194.95 and 168.09 ppm, referring to carbons of formyl and carboxylic groups (Figures S7 and S9). The remaining carbon of carbonyl signals of acid and amide appeared in the ^{13}C-NMR spectra of compounds **5** and **6** in the regions at δ = 162-99, 163.00, and 160.80, 160.82 ppm.

The methyl 5-phenyl-2-cyano-2,4-pentadieneoate (**8**) compound was synthesized by the condensation of cinnamaldehyde (**7**) with methyl cyanoacetate in the presence of a piperidine in situ methanol solvent. Finally, treatment of compound **8** with 1-*N*-azacoumarin-3-carboxylic acid (**3**) in dimethyl formamide, with the existence of triethylamine under reflux, produced the corresponding 1-*N*-[4-cyano-5-methoxy-5-oxo-1-phenylpenta 1,3-diene-1-yl)-azacoumarin-3-carboxylic acid (**9**) (Scheme 3).

The ^{1}H-NMR spectrum of compound **9** displayed two characteristic singlets at δ = 3.75 and 8.89 ppm, referring to the protons of methoxy (OCH_3) group and H-4 of -N-aza coumarin ring (Figure S10). Protons of aromatic rings, olefin, and hydroxy functions of the carboxylic group were observed in the ^{1}H-NMR spectrum of this compound in the region at δ = 7.24–8.44 ppm (Figure S10). The ^{13}C-NMR spectrum of compound **9** showed three new carbon signals at δ = 162.80, 103.39, and 53.32 ppm due to the carbons of carbonyl (ester), cyano, and methoxy (OCH_3) groups (Figure S11). Additionally, the carbon signals of two carbonyl groups in the 1-*N*-azacoumarin-3-carboxylic acid ring appeared at δ = 163.00 and 160.82 ppm. In addition, characteristic carbon signals due to the aromatic and olefinic groups resonated in the region at δ = 156.55–118.94 ppm (Figure S11).

2.2. Anti-Cancer Evaluation

2.2.1. In Vitro Cytotoxic Activity of the Synthesized Compounds Against MCF-7 Cell Line

The synthesized compounds **3**–**6** and **9** were assessed for their anti-cancer activity against MCF-7 using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to assess cell viability. Doxorubicin (Dox) was utilized as a reference drug control. The data are reported as compound concentrations causing a net 50% loss of initial cells (IC_{50}) (Table 1). The acquired data of the tested quinolinone derivatives **3**–**6** and **9** showed that compound **6** was the most effective compound against the MCF-7 cell line, with IC_{50} values of 2.56 ± 0.13, in contrast to Dox IC_{50} = 2.82 ± 0.07 μM. Moreover, compounds **5** and **6** revealed moderate cytotoxic activity with IC_{50} values of 10.13 ± 0.83 and 14.06 ± 0.63 μM, respectively (Table 1).

Table 1. In vitro anti-cancer activity of quinolinone derivatives 3–6 and 9.

Compound	IC_{50} Values (μM)/ MCF-7
3	>50
4	>50
5	10.13 ± 0.83 ^{b}
6	2.56 ± 0.13 ^{a}
9	14.06 ± 0.63 ^{b}
Dox	2.82 ± 0.07 ^{a}

Each value is the mean of three experiments ± SEM. Different superscript letters designate significant differences ($p < 0.05$) using Duncan's multiple range test.

2.2.2. Cell Cycle Analysis

To examine the mechanism underlying the cytotoxic activity of the most active compound (**6**), the cell cycle distribution profile of MCF-7 cells was measured. MCF-7 cells were incubated with compound **6** at a concentration of 2.56 μM for 48 h. As a result, the percentage of MCF-7 cells increased in the G2/M phase by 34.62% compared with control cells (12.04%) (Figure 2). This result suggested that compound **6** causes perturbations in cell cycle progression in the G2/M phase, leading to an inhibition of cell survival. Cell cycle and apoptosis play significant functions in the regulatory mechanisms of cell development and growth. The effect of many substances which are used as anti-cancer agents was found to be through arresting of the cell cycle during the phases G0/G1, S, and G2/M, which

stimulated and induced apoptosis [37–39]. Thus, we conclude that the effect of compound **6** on cancer cell line was through the disturbances of compound **6** in cell cycle, progression especially in the G2/M phase, suggesting an antimitotic effect.

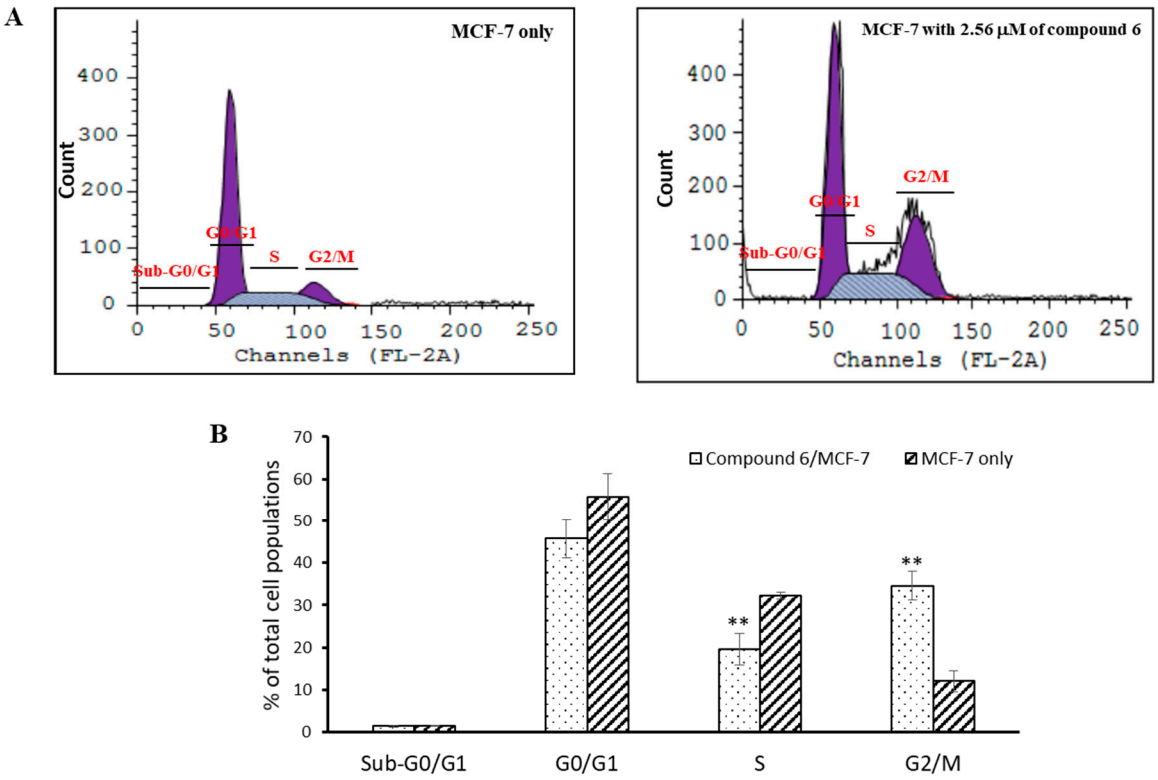


Figure 2. The effect of compound 6 at the concentration of 2.56 µM on the MCF-7 cell cycle progression. (**A**) Representative flow cytometry data of the cell cycle change. (**B**) Percentage of the total cell populations at each cell cycle phase in each cell. ** $p < 0.01$ according to Tukey's test.

2.2.3. Apoptosis Analysis

The apoptosis ratio was determined in MCF-7 cells after incubation with compound **6** for 48 h. The results exhibited that compound **6** could enhance the early apoptosis stage by 3.90% compared with the untreated cells (Figure 3). Moreover, compound **6** could increase the late apoptosis stage by 6.61% in comparison with the control group (Figure 3). These data indicate the involvement of compound **6** in the apoptosis process of MCF-7 cells. Thus, the flow cytometry investigation of MCF-7 cells indicated that the cancer cell tended to change from viable to apoptotic after the treatment with compound **6**.

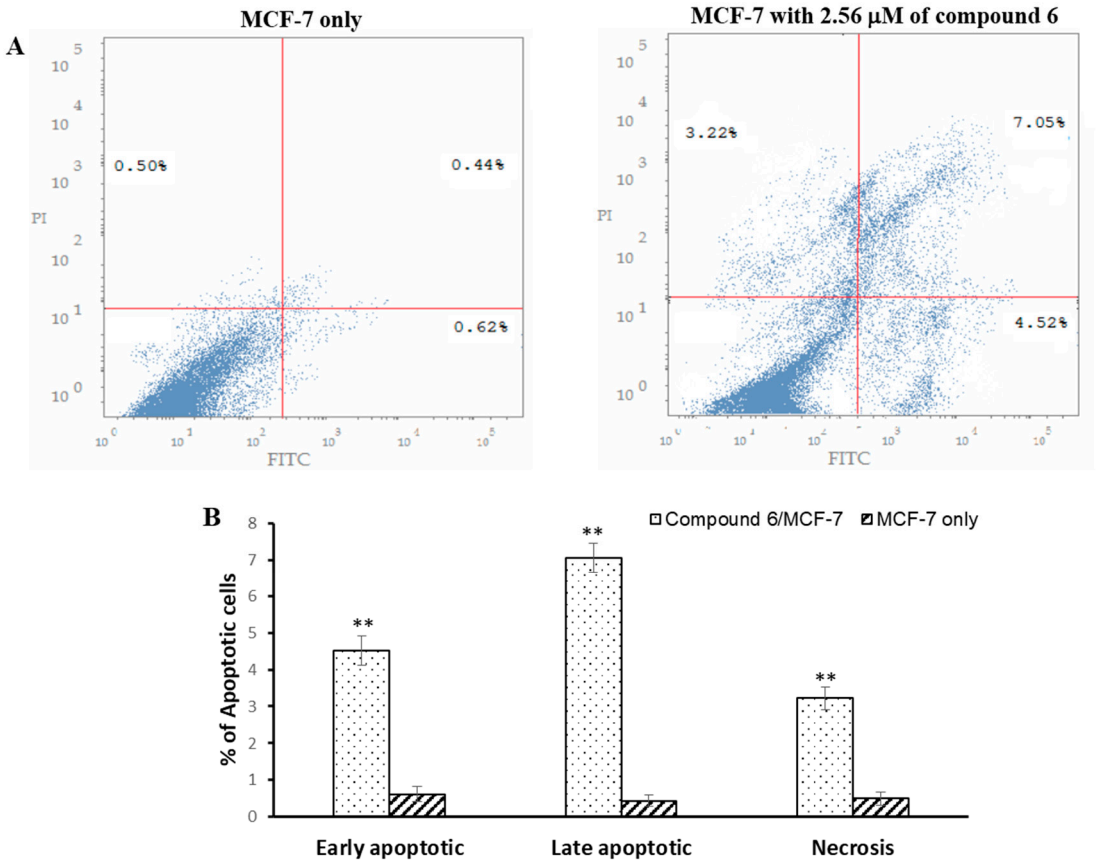


Figure 3. The apoptotic and necrotic cells obtained from MCF-7 cells alone or with compound 6 at a concentration of 2.56 µM. (**A**) The flow cytometry results. (**B**) the percentage of the apoptotic cells. ** $p < 0.01$ according to Tukey's test.

3. Materials and Methods

Shimadzu 470 Spectrometer apparatus with KBr pellets verified melting point apparatus (NP-D) and infrared spectra. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a Bruker 400 NMR MH_2 spectrometer in DMSO-d_6 and solution using TMS as an internal reference. Chemical shifts are given to δ-scale (ppm). The elemental analysis was performed on a Perkin-Elmer 2400 Series II CHN elemental analyzer. Chemicals used in syntheses were obtained from Aldrich, Merck, and Fluka chemical companies.

3.1. Synthesis

The reagent grade chemicals were obtained from commercial sources and purified by either distillation or recrystallization before use. The purity of synthesized compounds was checked by thin layer chromatography (TLC) on silica gel plate using benzene:ethyl acetate.

3.1.1. Ethyl Coumarin-3-carboxylate (2)

Ethyl coumain-3-carboxylate (**2**) was obtained as a starting material via the condensation of 2-hydroxy-benzaldehyde with diethyl malonate in a base catalyst according to previous studies [26], and was authenticated using NMR and IR spectroscopy.

3.1.2. 2-oxo-1H-Quinoline-3-carboxylic Acid (3)

The ester (compound **2**) (0.01 mole) was dissolved in ethanol (100 mL), anhydrous potassium carbonate (0.03 mole) was included, and this mixture was allowed to reflux for 30 min. The ammonia solution (20 mL, 36%) was added to the reaction mixture and then heated under pressure for 3 h. Next, the mixture was cooled by pouring it into ice water and neutralizing it with dilute hydrochloric acid (2%). The resultant solid was filtered off, washed with water, dried, and recrystallized from dimethylformamide to give a colorless

crystal, yield 63%, m.p. 285 °C IR (KBr) υ(cm^{-1}) = 3481 (br-OH), 3368 (NH), 1716, 1685 (C=O), 1605, 1563 (C=C), 1161, 1009 (C-O) cm^{-1}; ^{1}H-NMR (DMSO–d6) δ: 7.42–7.98 (m, 5 H, Ar–H and NH), 8.09 (br.s, 1H, OH), 8.87 (s, 1H, H-4 of azacoumarin ring); ^{13}C-NMR (DMSO–d_6) δ: 163.00 (C-11), 160.80 (C-2), 154.51(C-9), 148.26 (C-4), 134.58 (C-5), 130.74 (C-10), 125.56 (C-6), 119.78 (C-7), 118.93(C-8), 116.60 (C-3) ppm. Anal. Calcd. for C10H7NO3 (189): C, 63.49; H, 3.70; N, 7.41. Found: C, 63.18; H, 3.49; N, 7.21.

3.1.3. 1-*N*-(acetyl)-Azacoumarin-3-carboxylic Acid (4)

A solution of compound **3** at 0.01 mol was heated in a solution of acetic anhydride (25 mL) under reflux for 4 h. After that, it was allowed to cool by pouring it into ice water while stirring. The reaction mixture was left for 24 h, and then the precipitate formed was filtered and washed well with water and left to dry. Finally, the product was recrystallized from ethanol to produce 4 (Figure 4) colorless crystals; the yield was 58%, m.p. 170 °C. IR (KBr) υ(cm^{-1}) = 3428–3015 (br-OH), 1720, 1695 (C=O), 1608, 1583 (C=C), 1112, 1017 (C–O) cm^{-1}; 1H-NMR (DMSO–d6) δ: 2.38 (s, 3 H, CH3), 7.28–8.07 (m, 4H, Ar-H), 8.11 (br.s, 1H, OH), 8.69 (s, 1H, H-4 of azacoumarin ring) ppm; ^{13}C-NMR (DMSO–d6) δ: 190.59 (C-12), 169.79 (C-11), 157.67 (C-2), 150.42 (C-9), 136.21 (C-4), 133.12 (C-5), 131.44 (C-10), 129.2 (C-6), 127.13 (C-7), 126.20 (C-8), 124.12(C-3), 21.27 (C-13) ppm. Anal. Calcd. for C12H9NO4 (231): C, 62.34; H, 3.90; N, 6.06. Found: C, 62.11; H, 3.66; N, 5.88.

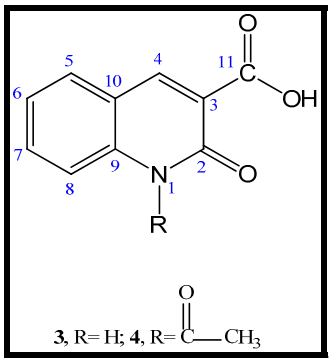


Figure 4. The structure of 1-*N*-(substituted)-azacoumarin-3- carboxylic acids (compounds **3** and **4**).

3.1.4. General Procedure for the Synthesis of 1-*N*-(substituted)-Azacoumarin-3-carboxylic Acids (**5**, **6**, and **9**)

A mixture of compound **3** (0.01 mole) and α,β-5-unsaturated carbonyl compounds (namely, cinnamaldehyde, cinnamic, acid and methyl 5-phenyl-2-cyano-2,4-pentadine oats (0.01 mole) in 50 mL dimethylformamide was refluxed for 4 h. After cooling, the resultant mixture was poured into water and neutralized with dilute hydrochloric acid (2%). Then, the resulting product was collected after filtration, washed, and dried. Finally, the final formed products were recrystallized from a methanol solvent to give compounds **5**, **6** and **9** (Figure 5).

3.1.5. 1-*N*-(2-Formyl-1-phenyl) Vinyl-azacoumarin-3-carboxylic Acids (**5**)

Compound **5** was produced as pale-yellow crystals with a yield of 63%, m.p. 187 °C. IR (KBr) υ(cm^{-1}) = 3430 (br.OH), 1725, 1689 (C=O), 1607, 1583 (C = C), 1181, 1093 (C–O) cm^{-1}; ^{1}H-NMR (DMSO –d_6) δ: 6.81–8.00 (m, 12 H, Ar-H olefinic-H and OH), 8.89 (s, 1H, H-4 of azacoumarin ring), 9.75 (s, 1H, CHO) ppm; ^{13}C-NMR (DMSO-d_6) δ: 194.95 (C-20), 162.99 (C-11), 160.80 (C-2), 154.51(C-9), 148.28 (C-4), 144.41(C-12), 134.62 (C-16), 134.57 (C-5), 131.70 (C-10), 130.73 (C-13) 129.56, 129.37 (C-15, 17),129.22, 128.64 (C-14,18), 125.56 (C-6), 119.78 (C-7), 119.68 (C-19), 118.93 (C-8), 116.60 (C-3) ppm. Anal. Calcd. C_{19} $H_{13}NO_4$ (319): C, 71.47; H, 4.10; N, 4.39. Found: C, 71.26; H, 3.83, N, 4.08.

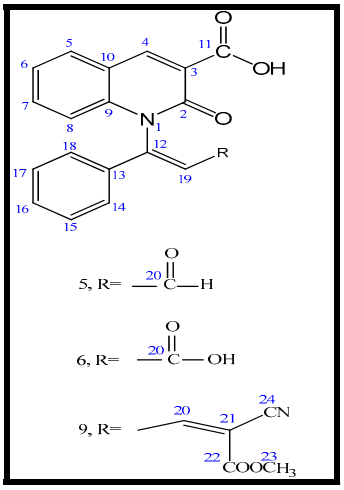


Figure 5. The structure of the derivatives **5**, **6,** and **9**.

3.1.6. 1-*N*-[2-(Hydroxy) Caybonyl-1-(phenyl) Vinyl]-azacoumarin-3-carboxylic Acid (**6**)

Compound **5** was produced as colorless crystals, with a yield of 73%, m.p. 233 °C. IR (KBr) υ(cm^{-1}): 3451–2985 (br. OH), 1717–1688 (C=O), 1605, 1563 (C=C), 1178, 1089, 1021 (C–O) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 6.54 (d, 1H, olefinic), 7.33–8.10 (m, l0 H, Ar-H and OH), 8.89 (s, 1H, H-4 of azacoumarin ring), 12.56 (br.s.,1H, OH) ppm; ^{13}C-NMR (DMSO-d_6) δ: 168.69 (C-20), 163.00 (C-11), 160.82 (C-2), 154.52 (C-9), 148.09 (C-4), 144.39 (C-12), 134-70 (C-16), 134.59 (C-5), 130-75 (C-10), 130.71 (C-13), 129.38 (C-15,17), 128.69 (C-14,18), 125.57 (C-6), 119.76 (C-7), 119.73 (C-19), 118.94 (C-8), 116.61 (C-3) ppm. Anal. Calcd. for $C_{19}H_{13}NO_5$ (335): C, 68.06; H, 3.91; N, 4.18. Found: C, 67.89; H, 3.58; N, 4.02.

3.1.7. 1-*N*-(4-Cyano-5-methoxy-5-oxo-1-phenylpenta-1,3-diene-1-y)-azacoumarin-3-cayboxylic Acid (**9**)

Compound **5** was produced as pale yellow crystals, with a yield of 62%, m.p. 198 °C. IR (KBr) υ(cm^{-1}) = 3483–3105 (br.OH), 2225 (CN), 1735–1689 (C=O), l606, 1583 (C=C), 1183, l083 (C–O) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.75 (s, 3H, OCH_3), 7.24–8.44 (m, 12 H, Ar–H, olefinic-H and OH), 8.89 (s, 1H, H-4 of azacoumarin ring) ppm; ^{13}C-NMR (DMSO-d_6) δ: 163.00 (C-11), 162.80 (C-22), 160.82 (C-2), 156.55 (C-12), 154.51 (C-9), 150.70 (C-13), 148.30 (C-4) 135.08 (C-15), 134.58 (C-5), 131.76 (C-16), 130.75 (C-10), 129.72 (C-15, 17), 129.19 (C-14, 18), 128.38 (C-19), 125.56 (C-6), 123.08 (C-21), 119.76(C-7), 118.94 (C-8), l03.39 (C-25), 53.32 (C-24) ppm. Anal. Calcd. for $C_{23}H_{16}N_2O_5$ (400): C, 69.00; H, 4.00; N, 7.00. Found: C, 68.83; H, 3.73; N, 6.86.

3.2. Anti-Cancer Assay

3.2.1. In Vitro Anti-Cancer Activity Against Breast Cancer Cell Line (MCF-7)

To investigate the effect of the synthesized compounds **3**–**6** and **9** as anti-cancer drugs, a cytotoxicity test was performed using the MTT technique. The cells were distributed in a 96-well plate with approximately 10^4 cells/well and then left for 24 h before adding the synthesized compounds to allow the cell to attach to the plate. After 24 h, different concentrations of the synthesized compounds were added to each cell monolayer. Each concentration had six wells. The 96-well plates were incubated at 37 °C in the presence of 5% CO_2. After two days, MTT was added with a final concentration of 0.5 mg/mL to each well, and the plates were incubated for an extra 4 h at 37 °C in the presence of 5% CO_2. Color intensity was measured using an ELISA reader. The correlation between survivor fracture and drug intake was drawn to obtain a survival curve for the MCF-7 cancer cell line after the specific concentration of the synthesized compounds. All experiments were performed in triplicates.

3.2.2. Cell Cycle Analysis

To study the effect of the synthesized compounds on the cell cycle, approximately 2×10^5/well MCF-7 cells were treated with compound **6** at its IC_{50} value for two days. After exposure to this process, cells were washed twice with ice-cold phosphate saline (PBS) and then cells were collected by centrifugation, followed by fixing of the cells in ice-cold 70% ethanol at 4 °C for 30 min. Cells were washed using 1× PBS solution at 37 °C for 30 min. After that, cells were collected using centrifugation at light speed (2000 rpm) for 5 min, and then cells were stained with a solution of propidium iodide at a final concentration of 1 µg/mL. The samples were mixed gently and left at room temperature in the dark for 20 min. DNA content was investigated via a BD FACSCanto flow cytometer (BD Biosciences Systems, San Jose, CA, USA). The results were analyzed using FACSDiva software (BD Biosciences Systems).

3.2.3. Apoptosis Determination by Annexin-V Assay

The MCF-7 cells (2×10^5/well) were incubated with compound 6 at its IC_{50} concentration value for 48 h. Next, cells were collected and washed twice with PBS at low speed (2000 rpm for 10 min at 4 °C). All cells were dissolved in 100 µL of a binding solution, and then 5 µL of Annexin V-FITC was added. Then, cells were incubated for 10 min at room temperature, additional 400 µL of binding solution was added. Next, cells were stained with PI stain and examined with a FACSCalibur flow cytometer (Becton and Dickinson, Heidelberg, Germany). The obtained results were analyzed using Cell-Quest software (Becton and Dickinson, Heidelberg, Germany).

3.2.4. Statistical Analysis

Statistical comparisons were measured by a one-way ANOVA with Duncan's test using IBM SPSS version 26. A probability level of 0.05 or lower was considered statistically significant; ** $p < 0.01$.

4. Conclusions

The medicinal properties of coumarin have been discovered due to its presence in various medicinal plants. Therefore, coumarin has different biological activities and has a positive effect on human health. The present study focused on the synthesis of substituted azacoumarin-3-carboxylic acids as 1-*N*-(acetyl)-azacoumarin-3-carboxylic acid (**4**), 1-*N*-(2-formyl-1-phenyl) vinyl-azacoumarin-3-carboxylic acids (**5**), 1-*N*-[2-(hydroxy) caybonyl-1-(phenyl) vinyl]-azacoumarin-3-carboxylic acid (**6**) and 1-*N*-(4-cyano-5-methoxy-5-oxo-1-phenylpenta-1,3-diene-1-y)-azacoumarin-3-cayboxylic acid (**9**). The chemical structures of these compounds were discussed based on the elemental analyses, infrared, and proton NMR spectroscopy. All the synthesized quinolinone derivatives were investigated for their cytotoxic activity against the MCF-7 cell line. Compound **6** was considered the most capable anti-cancer drug among the tested compounds. The mechanism of action was assessed using flow cytometric assay. The data revealed that the compound **6** was able to arrest the cell cycle in the G2/M phase and pre-G1 apoptosis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cryst11050565/s1, Figure S1: FT-IR spectrum of compound (3), Figure S2: ^{1}H-NMR spectrum of compound (3), Figure S3: ^{13}C-NMR spectrum of compound (3), ^{1}H-NMR spectrum of compound (4), Figure S5: ^{13}C-NMR spectrum of compound (4), Figure S6: ^{1}H-NMR spectrum of compound (5), Figure S7: ^{13}C-NMR spectrum of compound (5), Figure S8: ^{1}H-NMR spectrum of compound (6), Figure S9: ^{13}C-NMR spectrum of compound (6), Figure S10: ^{1}H-NMR spectrum of compound (9), Figure S11: ^{13}C-NMR spectrum of compound (9).

Author Contributions: Conceptualization, M.S.R. and I.M.E.-D.; methodology, I.M.E.-D., M.S.R. and A.G.; software, I.M.E.-D. and M.S.R.; validation, W.F.A., M.A. and A.S.A.; formal analysis, A.G., W.F.A., M.A., A.S.A. and M.S.R.; investigation, A.G., M.A., A.S.A. and M.S.R.; data curation, A.G., I.M.E.-D. and M.S.R.; writing—original draft preparation, I.M.E.-D. and M.S.R.; writing—

review and editing, A.G. and W.F.A.; supervision, W.F.A. and M.S.R.; project administration, A.G.; funding acquisition, A.G. and W.F.A. All authors have read and agreed to the published version of the manuscript.

Funding: The authors appreciate the Taif University Researchers Supporting Project (number TURSP-2020/39), Taif University, Taif, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the results of the present research are included in the article.

Acknowledgments: The authors are appreciative of the support of Taif University Researchers Supporting Project (number TURSP-2020/39), Taif University, Taif, Saudi Arabia.

Conflicts of Interest: The authors declare no conflict of interest.

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