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DESIGN, SYNTHESIS, AND CYTOTOXIC ACTIVITY OF SOME NOVEL N-(SUBSTITUTED) BENZAMIDE DERIVATIVES BEARING COUMARIN AND 1-AZOCOUMARIN COMPOUNDS

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ABSTRACT. Among oxygen-containing heterocyclic compounds such as coumarin and azacoumarin derivatives, the scaffold has become an important construction motif for developing new drugs. Coumarin and its derivatives possess many types of biological activities and have been reported to show significant cytotoxic activity. N-(6,8-disubstituted coumarin-3-yl)benzamides (**8a-c**) namely (3-N-(benzoyl) aminocoumarin-6-ylmethyl acetate (**8a**); N-[6-(1-acetylpyrazol-3-yldiazineyl) coumarin-3-yl] benzamide (**8b**); N-(8-methoxy-6-bromo-coumarin-3-yl) benzamide (**8c**), were synthesized via a cyclocondensation reaction of 5-(chloromethyl)-2-hydroxybenzaldehyde (**3**), 5-(pyrazol-3-yl-diazineyl)-2-hydroxybenzaldehyde (**4**), and 5-bromo-3-methoxy- 2-hydroxybenzaldehyde (**5**) with N-benzoylglycine (**7**), in good yield. Treatment of compound **8c** with ammonia in the presence of anhydrous potassium carbonate to yield N-(5-bromo-8-methoxy-1-azocoumarin-3-yl) benzamide (**9**). Compound (**9**) was acetylated with acetic anhydride to give N-(2-acetoxy-5-bromo-8-methoxyquinolin-3-yl) benzamide (**10**). N-(substituted coumarin and azacoumarin-3-yl) benzamides (**8-10**) were tested for their in vitro cytotoxic activity against (HepG2) cell line. Furthermore, DNA flow cytometry investigation over HepG2 cells indicated that compound **8a** demonstrated arrest at G1/S stages of the cell cycle and induction of apoptosis by rising pre-G1 stage. Compound **8a** displayed a significant tubulin polymerization inhibition.

KEY WORDS: Synthesis, Coumarin, Azacoumarin, Drugs, Cytotoxicity

INTRODUCTION

Cancer is one of the main health problems, it is known as the abnormal division and accumulation of cells in an organism and is one of the most common causes of disease-related deaths worldwide [1]. As a result of the increased number of deaths due to cancer, according to the World Health Organization (WHO) report, more than 13 million cancer deaths will happen in 2030 [2, 3], while the number of cancer deaths was estimated at 9.6 million deaths in 2018 [4, 5]. Cancer is a chief leading basis of mortality in developed and developing countries [6, 7]. Recently, about 150 species of coumarin have been found in 36 diverse plant families [8]. It has diverse, pharmacological activities [9-15] like anticancer. To target cancer, coumarin derivatives directly or indirectly inhibit the process of tubulin polymerization, mitosis, and DNA replication by inhibiting various enzymes like protein kinases, sulphatases, aromatase, caspases, and heat shock proteins [16-18]. The synthetic and semi-synthetic derivatives of natural products have been found to exhibit various biological activities. It has been established that heterocyclic compounds containing oxygen atoms play an important role in designing novel molecular architectures for medical use [19]. The amide linker between the coumarin and the side chain in the 3-position, such as N-(substituted)-6,8-disubstituted coumarin-3-carboxamides (I, II and III) exhibited good enzyme inhibitors (Structure 1) [20].

As part of our ongoing research into the discovery of the cytotoxic active coumarin phenylamide, in this work we attempt to investigate the effect of coumarinylamide benzene, another structural isomer (Structure 2) on cancer cell lines. The structural variations were explored

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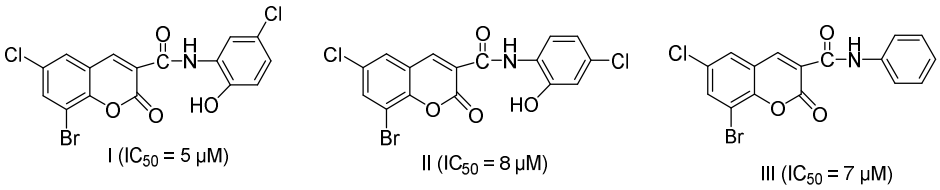
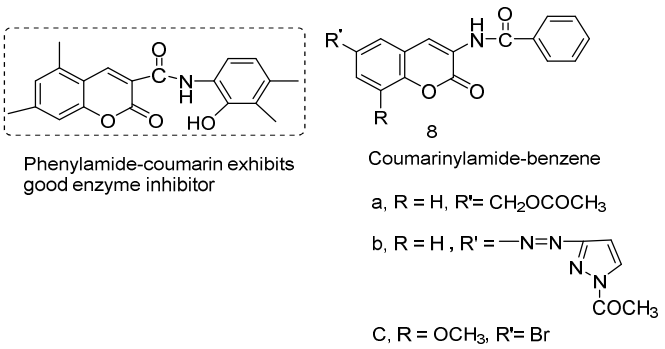
by placing substituted coumarin moiety in the position at the nitrogen atom of benzamide, and the synthesized coumarinylamide-benzene derivatives also exhibited antitumor activities [21-23]. Also, here in this paper has aimed the synthesis of quinoloinylamide-benzene and evaluated for their cytotoxic activity against liver cancer cell lines (HepG2).



Structure 1. Phenylamide-coumarins as enzyme inhibitors.



Structure 2. Design Scaffold.

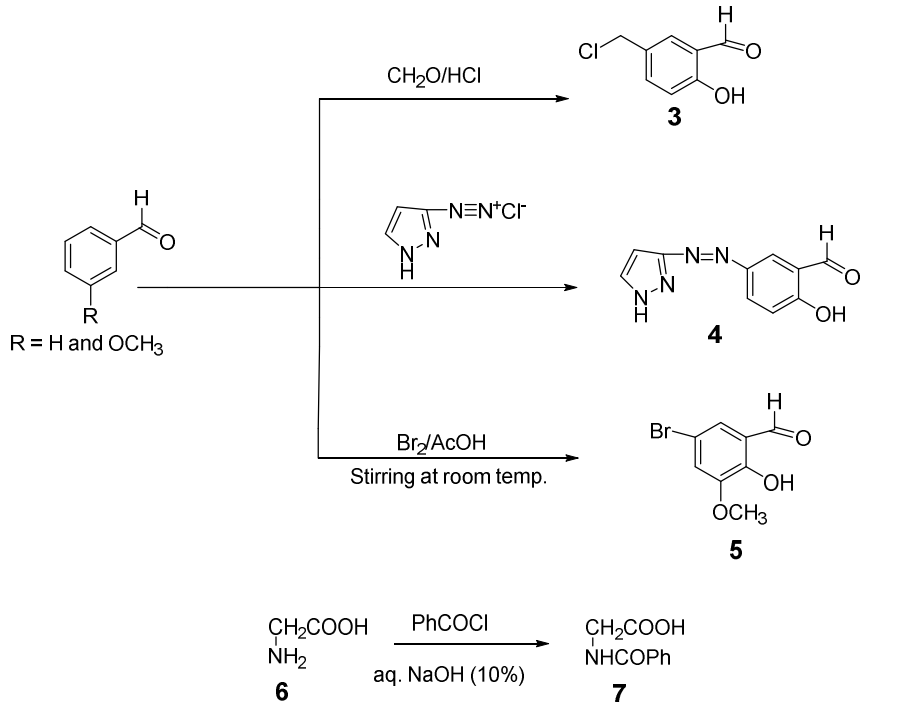
RESULTS AND DISCUSSION

Chemistry synthesis

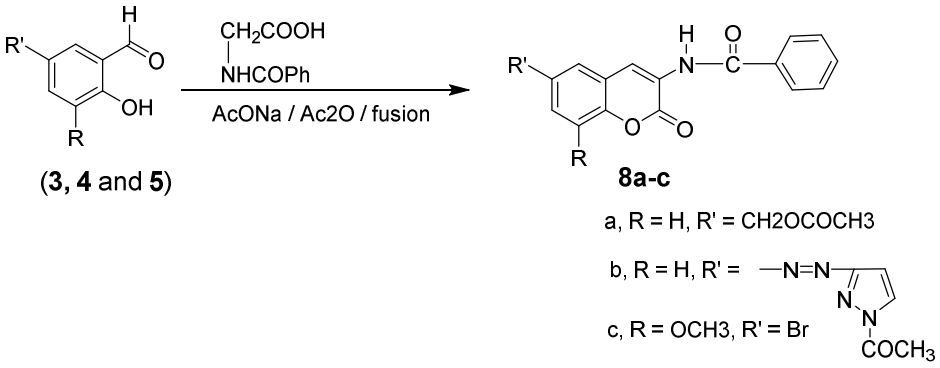
The synthetic pathway leading to the N-(substituted)benzamide derivatives containing coumarin and azocoumarin moieties (**8-10**) is outlined in Schemes 1, 2 and 3. The starting materials 5-(chloromethyl)-2-hydroxybenzaldehyde (**3**), 5-(pyrazol-3-diazenyl)-2-hydroxybenzaldehyde (**4**), and 5-bromo-3-methoxy-2-hydroxybenzaldehyde (**5**) were obtained via the chloromethylation and diazotization of 2-hydroxybenzaldehyde (**1**) with formaldehyde in the presence of hydrochloric acid and coupling diazonium chloride of 3-aminopyrazole, while the 5-bromo-3-methoxy-2-hydroxybenzaldehyde (**5**) was prepared via the halogenation of 3-methoxy-2-hydroxybenzaldehyde (**2**), with bromine in glacial acetic acid. Also, the hippuric acid (**7**) was formed via the reaction of glycine (**6**) with benzoylchloride in aqueous sodium hydroxide with stirring at 0-5°C (Scheme 1), according to literature procedures [24-26].

N-(substituted coumarin-3-yl) benzamides (**8a-c**) were synthesized via the cyclo-condensation of 2-hydroxy-benzaldehyde derivatives (**3**, **4,** and **5**) with hippuric acid and (**7**) in the presence of fused sodium acetate and acetic anhydride under fusion (Scheme 2). Treatment of N-(5-bromo-8-methoxycaumorin-3-yl) benzamide (**8c**) with ammonia in ethanol in the presence of anhydrous potassium carbonate under reflux Led to the formation of N-(8-methoxy-5-bromo-2-oxo-1,2-didydroquinolin-3-yl) benzamide (**9**). The structure of compound **9** was confirmed

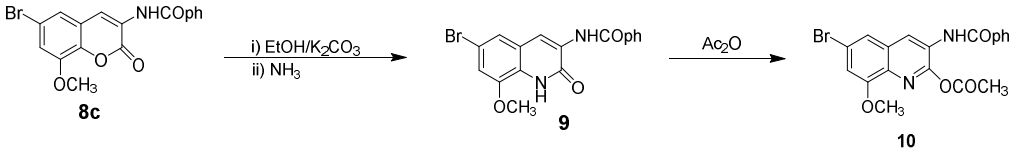
chemically via its acetylation with boiling acetic anhydride provided the corresponding N-(8-methoxy-5-bromo-2-acetoxy-quinolin-3-yl) benzamide (**10**, Scheme 3).



Scheme 1. Preparation of starting materials (**3**, **4**, **5**, and **7**) according to literature procedures.



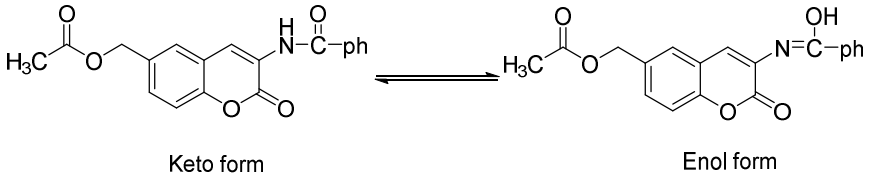
Scheme 2. Synthesis of N-(6,8-disubstituted coumarin-3-yl) benzamide derivatives (**8a-c**).



Scheme 3. Synthesis of N-(substituted quinoline-3-yl) benzamide derivatives (**9** and **10**).

*The spectroscopic methods investigation of some synthesized N-(substituted coumarin-3-yl and/or substituted azocoumarin-3-yl) benzamides (**8-10**)*

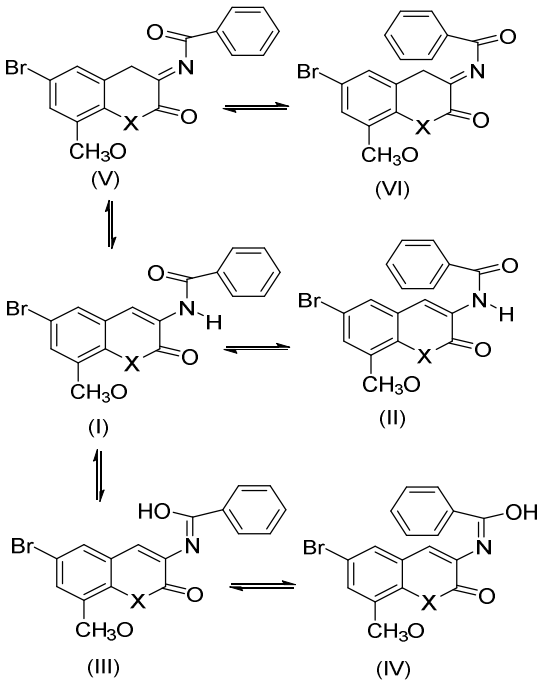
The structure of compounds (**8-10**) was determined and affirmed by several spectroscopic techniques including FT-IR, ^{1}H NMR, ^{13}C NMR, and Mass spectrometry (MS). The ^{1}H NMR spectrum of compound **8a** revealed that this compound presents in a tautomeric equilibrium (keto-enol tautomer, Structure 3).



Structure 3. Keto-Enol tautomers of compound **8a**.

The ^{1}H NMR spectrum of compound **8a** showed two singlet signals at δ, 2.10, 2.15 ppm due to the methyl protons (CH_3) and at δ 5.14, 5.25 ppm referring to the methoxy protons (OCH_3) which assigned to present the enol-keto tautomers. The ^{1}H NMR spectrum exhibited a singlet signal at δ 9.64 ppm, which indicated the NH proton of the keto form, which the proton of hydroxyl for the enol form (OH) appeared in the aromatic region because it is shielding via the formation of hydrogen bond with the carbonyl group of coumarin ring in the 'H NMR spectrum of compound **8a**.

The ^{13}C NMR exhibited signals of compound **8a** at δ 170.75, 170.32 ppm referring to the carbonyl of the ester of two isomers, at δ 167, 23, 160.40 due to the carbonyl of amide (NHCO) in two isomer forms, while the carbonyl (C=O) of coumarin ring appeared at δ 158.25 ppm. The mass spectrum of compound **8a** showed an intense molecular ion peak at m/z 337, corresponding to the molecular formula $C_{19}H_{15}NO_5$. The molecular ion peak at m/z 337 of compound **8a** underwent fragmentation to produce a base peak at m/z 105, corresponding to the benzoyl cation, followed by losing carbon monoxide (CO) to give an ion peak at m/z 77, indicated to the phenyl cation. These results affirm the successful cyclo-condensation and the formation of (3-(benzoyl) aminocoumarin-6-yl) methyl acetate (**8a**) scaffold Figures (1a, 1b, and 1c). From the current study, the ^{1}H, ^{13}C NMR spectra of compound **8c** showed the structure of this compound presented in tautomers and stereoisomers. In addition, the presence of two mixtures of isomers (51%: 49%) due to the isotopes of bromine atoms (Br^{79} and Br^{81}) are shown in Structure 4.



Structure 4. Different tautomers and stereoisomers of compounds **8c** and **9**.

The ^{1}H NMR spectrum of compound **8c** revealed the presence of four singlet singlets at δ 8.56, 8.57, 8.85, and 8.94 ppm assigned to the H-4 of coumarin ring, which indicated the formed four isomers of compound **8c** (I, II, III and IV). Also, the ^{1}H NMR spectrum of compound **8c** showed two singlet signals at δ 2.35 and 2.40 ppm due to the methylene protons (CH_2) in position-4 in the coumarin ring for two isomers (V and VI). In addition, the methoxy (OCH_3) group in compound **8c** appeared at δ 3.85, 3.87, 3.88, 3.92, and 3.94 ppm as singlet signals in the ^{1}H NMR of compound **8c**, due to the presence of six isomers of these compound. Also, the aromatic protons of compound **8c** were observed in the region at δ 7.05-8.10 due to the seven protons of the aromatic ring for six isomers. Additionally, the ^{13}C NMR spectrum of compound **8c** confirmed the presence of tautomers and stereoisomers. The ^{13}C NMR spectrum of compound **8c** showed five carbon signals of the methoxy (OCH_3) group at δ 57.38, 57.20, 57.03, 56.74, and 56.53 ppm, and the two carbon signals at δ 20.70 and 20.67 ppm due to the methylene (CH_2) group in the coumarin ring at position-4 for the two isomers (V and VI). The mass spectrum of compound **8c** showed intense molecular ion peaks at m/z 375 (M^{+}+2) and 373. (M^{+}), corresponding to the molecular formula $C_{17}H_{12}BrNO_4$. The molecular ion peak at m/z 373 underwent broken to produce a stable ion peak at m/z 105, corresponding to benzoyl cation (C_6H_5CO), It furthers the loss of carbon monoxide (CO) to give a peak at m/z 77.

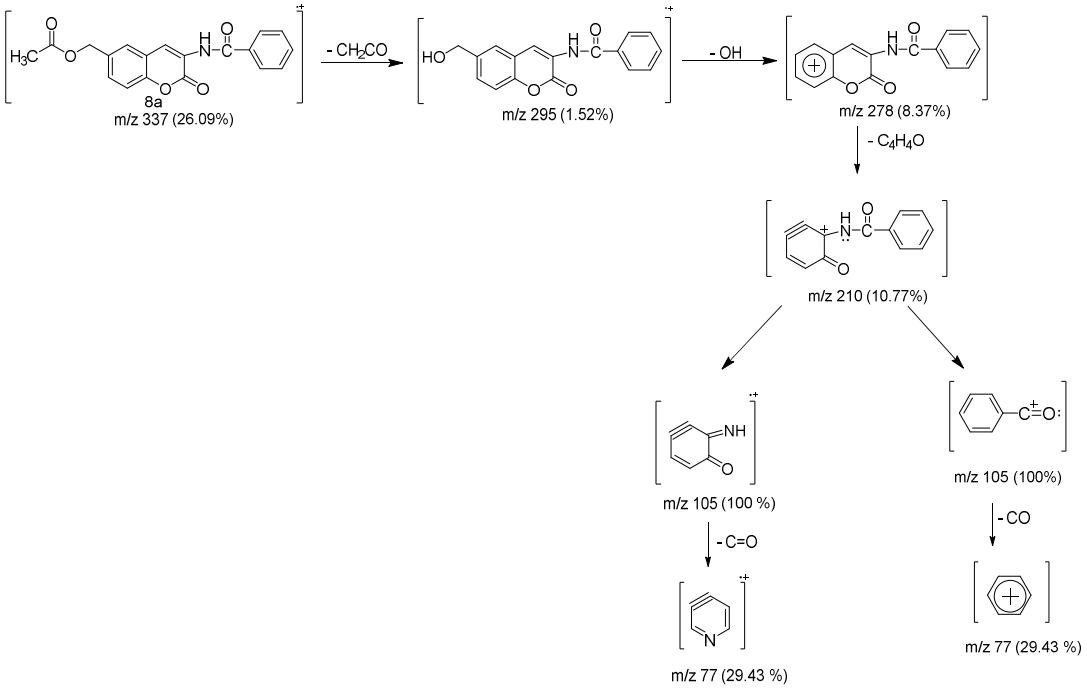
Similarly, the ^{1}H NMR of compound **9** exhibited two singlet signals at δ 2.74 and 2.90 ppm due to the methylene protons (CH_2) in the quinoline ring at position-4 for the two isomers and three singlet signals at δ 8.56, 8.87 and 8.97 ppm refer to the H-4 of quinoline ring for the four isomers of compound **9**. The NH proton signals appeared at δ 9.72, 9.77, and 9.80 ppm assigned to CONH for the three isomers as singlet signals of compound **9**. Also, the ^{1}H NMR spectrum of compound **9** showed five singlet signals at δ 3.77, 3.79 3.87, 3.94, and 3.96 ppm due to the

methoxy (OCH_3) group, which presented in the six isomers for compound **9**. The NH signal of quinoline appeared in the ^{1}H NMR of compound **9** at δ 10.25 ppm as a singlet signal. In addition, the aromatic protons appeared in the region at δ 7.06-7.98 ppm for the six isomers of compound **9**. The ^{13}C NMR spectrum of compound **9** exhibited two carbon signals at δ 39.68 and 39.26 ppm due to the methylene group (CH_2) in the quinoline ring at position-4 of two isomers, and at δ 57.04, 56.79 and 56.75 ppm refer to the methoxy (OCH_3) group of six isomers.

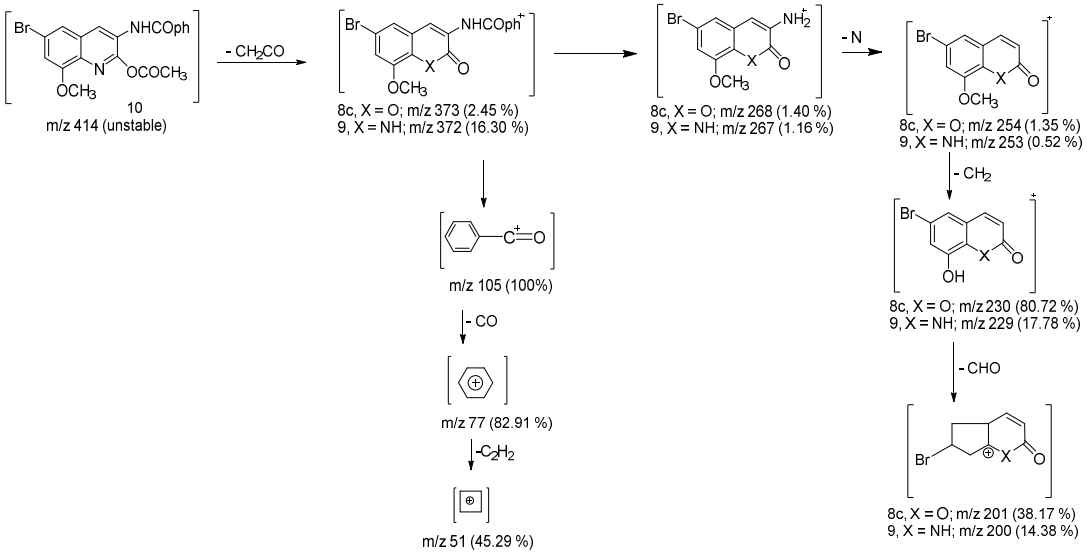
The ^{1}H NMR of compound **10** exhibited a new singlet signal at δ 2.40 due to the acetyl (CH_3CO) group, with the disappearance of the NH signal at δ 10.23 of compound **9**. The ^{13}C NMR spectrum of compound **10** exhibited two carbon signals at δ 168.72 and 20.66 ppm due to the acetyl group ($COCH_3$). These results confirmed the formation of compound **10** via the acetylation of compound **9** with acetic anhydride.

Mass spectrometry study

The mass spectral decomposition modes of the synthesized N-(substituted coumarin and/or substituted azacoumarin-3-yl) benzamide derivatives (**8-10**) have been investigated. The mass spectra of these compounds (**8-10**) showed the stable ion peak at m/z 105 of this compound. The mass spectrum of compound **8a** showed an intense molecular ion peak at m/z 337, corresponding to the molecular formula $C_{19}H_{15}NO_5$. The molecular ion of compound **8a** (Scheme 4) underwent fragmentation with rearrangement to produce a peak at m/z 295 by losing the ketene (CH_2CO) molecule. The loss of the hydroxyl (OH) group from the ion with m/z 295 resulted in an ion at m/z 278. The ion of m/z 278 underwent loss of cyclo-buta-2-ene-1-one (C_4H_4O) to give a peak at



Scheme 4. Main fragmentation pathway of compound **8a**.



Scheme 5. Main fragmentation pathway of compounds **8c**, **9** and **10**.

m/z 210, which further broke to give an ion at m/z 105. Ion of m/z 105 fragmented to give an anion of m/z 77 which lost carbon monoxide (CO). The mass spectra of compounds **8c**, **9**, and **10** are fully consistent with assigned structures. In most cases, intense, molecular ion peaks were observed at m/z 373 and m/z 372 for compounds **8c** and **9**, while the molecular ion peak of compound 10 is unstable. The molecular ion peak at m/z 414 (unstable) underwent HC≡CH broking to give an ion peak at m/z 372, corresponding to the molecular ion for the peak of compound **9**. The molecular ion of compounds **8c** and **9** (Scheme 5) underwent fragmentation to produce a stable ion peak at m/z 105. It further underwent loss of CO and to give peaks at m/z 77 and 51, respectively. Also, the molecular ion peaks at m/z 373 and 372 for the compounds **8c** and **9** underwent loss of benzoyl cation (C_6H_5CO) to give peaks at m/z 268 and 267. The ions at m/z 268 and 267 underwent loss of nitrogen atom, methylene (CH_2), and formyl (CHO) groups to produce peaks at m/z 254, 253; 230, 229, and 201, 200, respectively.

Cytotoxic activity

In vitro cytotoxic activity against Liver cancer cell line

The fabricated N-(substituted coumarin-3-yl) benzamides (**8a-c**) and N-(substituted azacoumoin-3-yl) benzamides (**9** and **10**) for their antiproliferative effect against human Hepatocellular Carcinoma cell line (HepG2). Table 1 are listed the IC_{50} values of the synthesized compounds **8a-c**, **9,** and **10**. Generally, it was noted that all the most newly fabricated components showed efficient antiproliferative effects toward the examined liver cytotoxic cell lines. The obtained results indicated that compound **8a** exhibited the most potent cytotoxic activity.

Cell cycle assay

Cell cycle analysis by quantitation of DNA content was one of the earliest applications of flow cytometry. The RNA of mammalian, yeast, plant, or bacterial cells can be stained by a variety of

DNA-binding dyes. The cytotoxic action of the most active compound was further discovered motivated by the preliminary antiproliferative assessment results. The most promising compound **8a** was, thus selected to examine the influence on the cell cycle analysis. In this study, HepG2 cells were incubated with the IC_{50} dose of compound **8a** for 24h. As illustrated in Figure 1.

Table 1. In vitro antitumor activity of compounds **8a-c**, **9,** and **10** over HepG2 cell lines data are expressed as the mean ± SD.

Compound No.	IC_{50} values (µg/mL) of HepG2
8a	211.16 ± 1.6
8b	241.5 ± 2.69
8c	299.21 ± 0.73
9	351.01 ± 4.60
10	231.94 ± 5.21

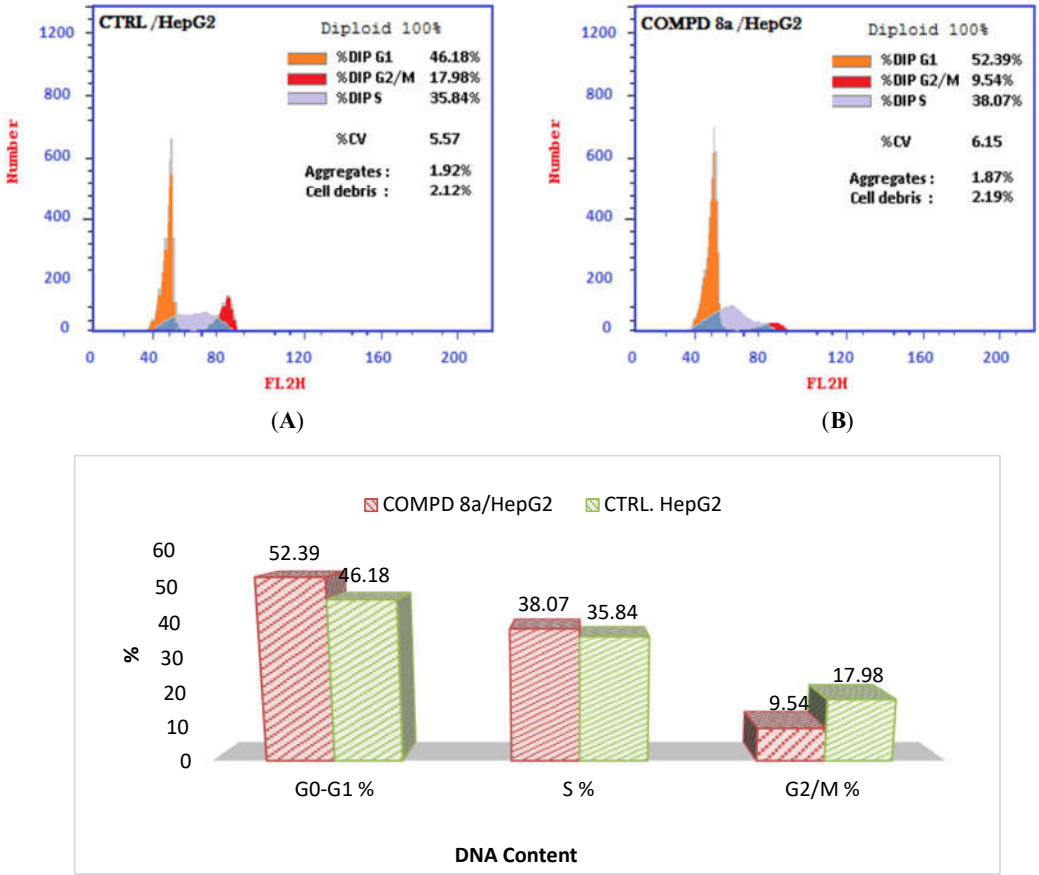


Figure 1. (**A**) Cell cycle distribution after compound **8a** treatment against HepG2 cell lines at its IC_{50} (µM); (**B**) Graphical representation of cell cycle analysis profile of compound **8a** against HepG2 cell lines.

Annexin V-FITC/PI screening

The prepared compound **8a** had a probable antitumor effect on the HepG2 cell propagation capability of the target compound to persuade apoptosis in HepG2 cells was detected using an exact assay. Annexin V-FITC/PI apoptosis necrosis method. As illustrated in Figure 2.

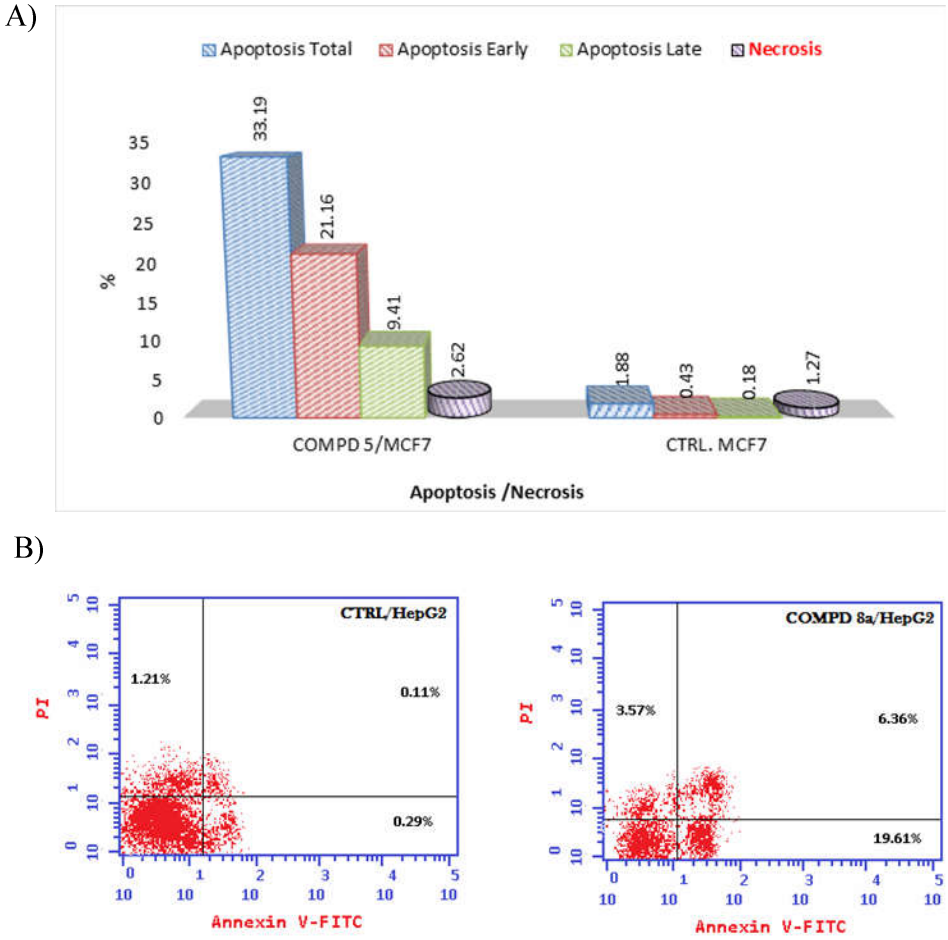


Figure 2. **A**) The percentage of apoptosis and necrosis caused by compound **8a** using HepG2 cell lines; **B**) Representative dot plots of HepG2 cells treated with compounds **8a** and at its IC50 for 24 h analyzed using flow.

Compound **8a** increased the apoptosis percentages at the total stage from 1.61% to 29.54% and at the early stage from 0.29% to 19.61% compared to the control one. Furthermore, the apoptosis percentage at the late phase amended from 0.11% to 6.36% compared to the control. Therefore, the tested compound **8a** could be considered a significant inducer of apoptosis in the HepG2 cell line.

Tubulin polymerization assay

This assay is an economical one-step procedure for determining the effects of drugs or proteins on tubulin polymerization. It is an adaptation of an assay originally described by Naik *et al.* [35]. The activity of compound **8a** was measured at different concentrations (100, 10, 1, 0.1, 0.01 µg/mL) against tubulin polymerization. Vinblastine is used as a standard drug for comparison purposes, and the results are given in Table 2. It was found that compound **8a** showed moderate activity with an IC_{50} value of 10.75 (Figure 3).

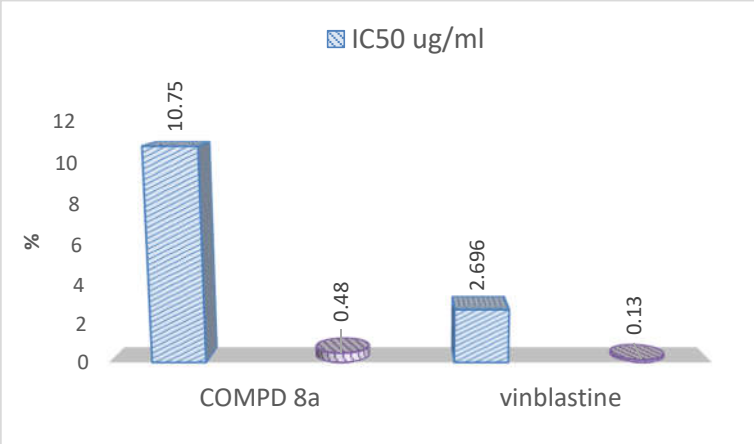


Figure 3. In vitro inhibition percentage of B-tubulin polymerization for compound **8a** at its IC_{50} compared to the control inhibition vinblastine.

Table 2.Tubulin polymerization inhibitory activity of compound **8a**.

Compound No.	K-Activity					IC_{50} values (µg/mL)
	100	10	1	0.1	0.01	
8a	10.548	34.98	68.66	83.84	105.73	10.75 ± 0.48
Vinblastine	6.672	17.55	37.59	68.58	101.23	2.69 ± 0.13

Molecular docking study

Molecular docking is a crucial element when designing computer-assisted drugs. Molecular docking is defined as getting an optimized modification for both the protein and drug with relative direction between them such that the free energy of framework 15 is minimized [27-30]. In this context, compound **8a** was docked into lipid-binding protein (2H80). The verified protocol was employed to accomplish the molecular docking simulation of N-(substituted coumarin)benzamide and the acquired data were analyzed to assess the best-interacting conformers, affinity scores, and mode of interaction (H-binding, hydrophobic and arene-arene interactions). Table 3 explains the affinity scores and the different types of interactions of compound **8a** with the active side of lipid-binding protein (2H80). The results showed a possible arrangement between compound **8** and receptor (2H80). Furthermore, agreeing with these results, the interaction between the lipid binding protein (2H80) receptor and compound **8a** is possible. A two-dimensional (2D) plot curve of docking with compound **8a** is shown in Figure 4.

Table 3. 2D interaction of compound **8a** to the active position of 2H80.

Structure	Compound **8a**	Receptor	Amino acid	Interaction	Distance (A)	E (kcal/mol)
A	N 26	OE1	GLU 20	H-donor	2.76	-3.0
	O 29	N	VAL 12	H-acceptor	3.15	-2.20
	6-ring	CB	ARG 69	Pi-H	3.90	-0.80
B	N 26	OE1	GLU 20	H-donor	2.88	-4.9
	O 29	NH_2	VAL 12	H-acceptor	3.12	-0.70
	6-ring	CD	ARG 69	Pi-H	4.20	-0.60

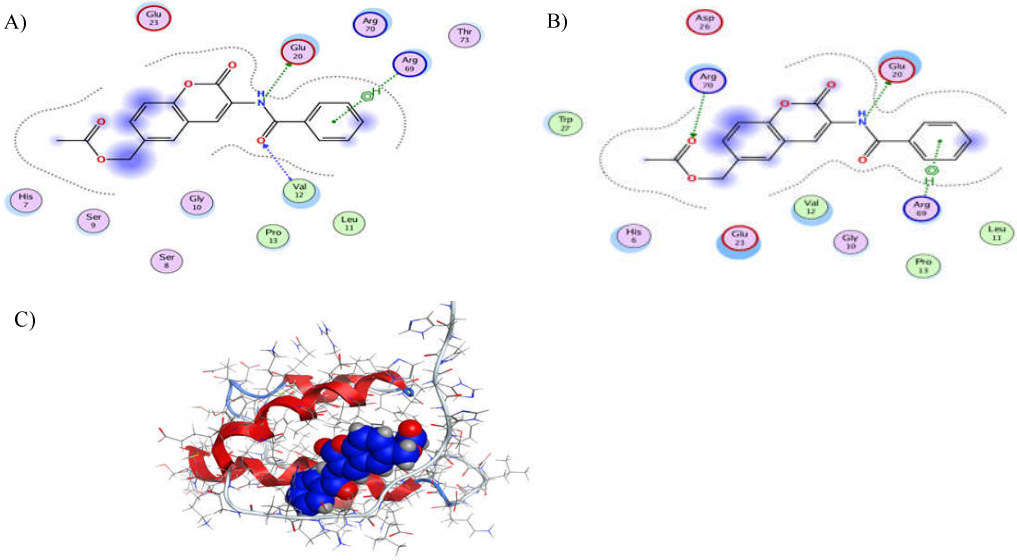


Figure 4. Interactive conformations (2D) and binding mode (3D) of compound **8a** in the active lipid binding protein (2H80).

EXPERIMENTAL

Chemistry

The chemical structure of N-(substituted coumarin and/or azocoumarin-3-yl) benzamides was characterized by elemental analysis, melting point, IK, Mass spectrometry, and ^{1}H and ^{13}C-NMR spectra analyses. The melting point of synthesized compounds was determined on an electrothermal 200 digital. IR spectra were measured as KBr discs utilizing Shimadzu 470 spectrophotometer (Kyoto, Japan), the NMR analysis was performed for a solution in deuterated solvents (DMSO-d6) utilizing a Bruker 400 DRX-Avance spectrometer at 400 MHz and 100 MHz for ^{1}H-NMR and ^{13}C-NMR, respectively. The chemical shifts are recorded in ppm utilizing tetramethyl silane (TMS) as the internal standard. The mass spectrometry analysis was performed utilizing Finnigan MAT SSQ-700 mass spectrometry operating at 70 eV. The elemental analysis was recorded on a Perkin-Elmer 2400 series (Haan, Germany). All commercially available chemicals were of analytical grade and were acquired from TCI, Alfa Aesar, and Sigma Aldrich unless otherwise specified.

*Synthetic procedures of N-(substituted coumarin-3-yl) benzamides (**8a-c**).*

A mixture of appropriate 2-hydroxybenzaldehyde derivatives (namely, 5-(chloromethyl)-2-hydroxybenzaldehyde (**3**), 5-(pyrazol-3-yl-diazineyl)-2-hydroxybenzaldehyde (**4**), and 5-bromo-3-methoxy-2-hydroxybenzaldehyde (**5**), 0.01 mol), N-benzoylglycine (0.01 mol), fused sodium acetate (0.03 mol) and acetic anhydride (5 mL) were fused on a hot-plate for 3-5 min. The reaction mixture was heated in a water bath under reflux for 2-3 h, then cooled and poured into the water with stirring. The precipitate formed was filtered off, washed with water, and dehydrated. The crude product was finally recrystallized from an appropriate solvent to afford compound **8**.

*(3-N-(benzoyl) aminocoumarin-6-ylmethyl) acetate (**8a**)*

The entitled compound **8a** was obtained as colorless crystals, yield 71%, m.p. 218 °C. IR (KBr) ν_{max}: 3394 (NH), 1725-1715 (br. C=O of ester and coumarin ring), 1673 (C=O of amide), 1580, 1529 (C=C), 1254, 1090, 1014 (C-O) cm^{-1}. ^{1}H NMR (DMSO-d6) δ: 2.10, 2.15 (s, 3H, $COCH_3$ of two isomers), 5.14, 5.25 (s, 2H, OCH_2 of two isomers), 7.43-7.45 (d, 2H, J = 8.0 Hz, Ar-H), 7.50 -7.64 (m, 3H, Ar-H), 7.76 (s, 1H, Ar-H), 7.96-7.98 (dd, 2H, J = 7.6 Hz, Ar-H), 8.62 (s, 1H, H-4 of coumarin ring), 9.64 (br. s, 1H, NH) ppm. ^{13}C NMR (DMSO-d6) δ: 170.75, 170.32, 167.23, 166.40 (C=O), 158.25 (C=O of coumarin ring), 150.25 (C-O), 134.06, 133.91, 133.64, 133.42, 132.80, 132.04, 130.41, 130.22, 129.15, 128.90, 128.11, 127.93, 127.74, 127.01, 126.78, 124.92, 119.71, 116.63 (C-aromatic and pyrane rings of two isomers), 65.52, 65.10 (OCH_2 of two isomers), 21.20 ($COCH_3$) ppm. MS: M/z (%) = 338 (M^++1, 2.54), 337 (M^+, 26.09), 336 (M^+-1, 2.83), 295 (1.52), 279 (1.25), 278 (8.37), 212 (3.07), 210 (10.77), 209(1.70), 168 (1.56), 111 (1.05), 106 (11.80), 105 (100), 104 (14.07), 103 (5.37), 77 (29.43), 76 (3.46). Anal. calcd. for $C_{19}H_{15}NO_5$ (M.wt = 337): C, 67.65; H, 4.45; N, 4.15. Found: C, 67.38; H, 4.25; N, 3.98.

*N-[6-(1-acetylpyrazol-3-yldiazineyl) coumarin-3-yl] benzamide (**8b**)*

The entitled compound **8b** was obtained as a pale-yellow solid, yield 69%, m.p. 205 °C. IR (KBr) ν_{max}: 3295 (NH), 1715, 1678 (C=O), 1605, 1583 (C=C), 1121, 1032 (C-O) cm^{-1}. ^{1}H NMR (DMSO-d6) δ: 2.21, 2.43 (s, 2H, CH_2 of coumarin ring of two isomers), 2.72, 2.80, 2.87, 2.93 (s, 3H, $COCH_3$ of four isomers), 7.49-8-39 (m, 10H, Ar-H and pyrazole ring for different isomers), 8.47, 8.79 (s, 1H, H-4 of coumarin of two isomers), 9.77 (s, 1H, NH), 10.41 (br. s, 1H, OH) ppm. MS: m/z (%) = 401 (M^+, unstable), 307 (1.04), 306 (1.53), 266 (1.67), 265 (5.03), 264 (2.34), 263 (1.04), 256 (1.24), 245 (1.23), 244 (4.65), 243 (28.76), 236 (1.05), 232 (1.15), 226 (1.06), 220 (1.17), 219 (1.36), 205 (3.70), 204 (3.19), 203 (2.67), 202 (16.90), 201 (46.22), 200 (4.45), 199 (4.50), 198 (1.55), 190 (1.63), 189 (1.10), 188 (1.63), 187 (7.66), 186 (15.59), 185 (3.33), 180 (2.02), 179 (1.14), 178 (2.50), 176 (1.06), 163 (5.07), 162 (32.59), 161 (4.31), 160 (13.32), 159 (99.53), 158 (9.11), 157 (1.79), 156 (2.06), 150 (1.09), 149 (4.95), 145 (11.08), 144 (67.46) , 143 (16.45), 142 (2.50), 135 (7.77), 134 (6.16), 133 (2.84), 132 (8.37), 131 (5.15), 130 (15.58), 129 (3.23), 128 (4.69), 127 (2.17), 118 (4.02), 117 (12.10), 116 (5.90), 115 (15.95), 114 (3.95), 106 (8.51), 105 (100), 104 (31.31), 103 (15.49), 102 (22.13), 91 (2.60), 90 (6.29), 89 (8.30), 88 (2.75), 87 (1.96), 77 (55.85), 76 (23.22), 75 (7.31), 74 (5.50), 64 (5.85), 63 (7.32), 62 (2.79), 60 (8.77), 51 (18.82), 50 (9.28). Anal. calcd. for $C_{21}H_{15}N_5O_4$ (M.wt = 401): C, 62.84; 3.74; N, 17.46. Found: C, 62.62; H, 3.51; N, 17.22.

*N-(8-methoxy-6-bromo-coumarin-3-yl) benzamide (**8c**)*

This compound was obtained as a pale orange solid, yield 72%, m.p. 245 °C. IR (KBr) ν_{max}: 3266 (NH), 1721, 1682 (C=O), 1608, 1583 (C=C), 1122, 1098, 1021 (C-O) cm^{-1}. ^{1}H NMR (DMSO-d6) δ: 2.35, 2.40 (s, 2H, CH_2 of coumarin ring of two isamors), 3.85, 3.87, 3.88, 3.92, 3.94 (s, 3H,

OCH_3 of five isomers), 7.05 (s, 1H, Ar-H), 7.06-7.78 (m, 4H, Ar-H), 7.95-7.97 (d, 1H, J = 8.00 Hz, Ar-H), 8.08-8.10 (d, 1H, J = 8.00 Hz, Ar-H), 8.56, 8.57, 8.85, 8.94 (s, 1H, H-4 of coumarin ring of four isomers), 9.68, 10.64, 10.23 (s, 1H, NH of three isomers). ^{13}C NMR (DMSO-d6) δ: 190.30, 189.44, 168.90, 168.74, 166.74, 166.41, 165.10, 157.54 (C=O of six isomers), 153.41, 152.41, 150.70, 147.6, 140.4, 139.23, 139.00 (C-O and C-4 of coumarin ring), 136.30, 134.73, 133.77, 132.86, 130.38, 129.97, 129.85, 129.15, 128.97, 128.84, 128.80, 128.74, 128.67, 128.19, 128.13 127.69, 125.78, 125.41, 125.19, 124.03, 123.65, 123.53, 121.90, 121.77, 121.64, 120.18, 120.06, 119.70, 119.42, 118.47, 117.14, 115.72, 110.92 (C-aromatic and pyranone ring of different isomers), 57.38, 57.20, 57.63, 56.74, 56.53 (OCH_3 of six isomers), 20.70, 20.67 (CH_2 of 4-hydropyranone of two isomers). MS: m/z (%) = 375 (M^++2, 2.91), 374 (M^++, 1.48), 373 (M^+, 2.45), 322 (1.83), 306 (1.04), 295 (1.29), 294 (1.16), 286 (1.61), 285 (1.02), 284 (1.75), 2.79 (1.71), 265 (1.94), 264 (2.75), 263 (1.27), 259 (1.08), 258 (1.11), 256 (1.35), 253 (1.09), 248 (1.46), 248 (1.46), 244 (3.92), 243 (11.77), 242 (2.11), 233 (17.17), 232 (86.68), 231 (40.20), 230 (32.68), 229 (80.72), 228 (8.80), 227 (2.32), 220 (6.88), 219 (4.98), 218 (4.54), 217 (2.22), 216 (4.23), 215 (4.12), 214 (6.69), 213 (3.89), 205 (4.06), 203 (11.99), 202 (24.90), 201 (38.17), 200 (15.59), 199 (9.94), 198 (3.98), 194 (5.83), 193 (2.24), 189 (16.31), 188 (7.49), 187 (32.60), 186 (52.17), 185 (20.76), 184 (38.81), 183 (17.46), 182 (6.40), 175 (3.02), 174 (3.71), 173 (4.79), 172 (5.97), 171 (3.88), 170 (4.92), 167 (5.67), 162 (17.80), 161 (18.97), 159 (74.49), 158 (11.47), 157 (5.74), 154 (5.05), 153 (8.27), 152 (18.61), 150 (7.93), 145 (9.49), 144(22.92), 143 (10.07), 136 (5.05), 134 (6.77), 133 (9.19), 131 (4.67), 130 (4.92), 122 (6.34), 121 (4.67), 108 (14.39), 106 (15.83), 105 (100), 104 (14.04), 102 (5.80), 79 (19.91), 78 (4.77), 77 (43.03), 76 (9.69), 74 (14.99), 65 (8.37), 63 (24.92), 62 (11.48), 60 (13.00), 51 (45.29), 50 (17.49). Anal. calcd. for $C_{17}H_{12}BrNO_4$ (M.wt = 373): C, 54.69; H; 3.22; N, 3.75. Found: C, 54.36; H, 3.03; N, 3.46.

*Synthesis of N-(8-methoxy-6-bromo-2(1H)-oxo-quinolin-3-yl)benzamide (**9**)*

A solution of compound **8c** (0.01 mole) in ethanol (30 ml) in the presence of anhydrous potassium carbonate (0.03 mol) was heated under reflux for 30 min. The reaction mixture was added to ammonia solution (10ml, 36%) and heated under reflux for 3 h then cooled and poured into water. The reaction mixture was neutralized with dilute hydrochloric acid (2%), and the solid formed was separated by filtration, washed with H_2O, dried, and recrystallized from ethanol to give compound **9** as yellow solid, yield 63%, m.p. 196 °C. IR (KBr) ν_{max}: 3310, 3208 (NH), 1698-1679 (br. C=O), 1605, 1589 (C=C), 1068, 1024 (C-O) cm^{-1}. ^{1}H NMR (DMSO-d6) δ: 2.74, 2.90 (s, 2H, CH_2 of 4-hydroquinoline of two isomers), 3.77, 3.79, 3.89, 3.94, 3.96 (s, 3H, OCH_3 for five isomers), 7.06, 7.11 (s, 1H, Ar-H), 7.19-7.21 (d, 1H, J = 8.00 Hz, Ar-H), 7.23 (s, 1H, Ar-H), 7.33 (s, 1H, Ar-H), 7.35-7.40 (t, 1H, J = 8.00 Hz, Ar-H), 7.50-7.66 (m, 3H, Ar-H), 7.93-7.95 (d, 1H, J = 8.00 Hz, Ar-H), 7.96-7.98 (d, 1H, J = 8.00 Hz, Ar-H) of six isomers of aromatic protons), 8.58, 8.87, 8.97 (s, 1H, H-4 of quinolinone of three isomers), 9.72, 9.77, 9.80 (s, 1H, NH of three isomers), 10.23, 10.24 (s, 1H, NH of two isomers) ppm. ^{13}C NMR (DMSO-d6) δ: 190.33, 167.23, 166.69, 166.45, 162.85, 157.56 (C=O and N=C-O), 150.73, 150.31, 149.20, 149.15, 147.64, 146.67, 145.30, 144.80, 140.31 (C-O, C=N, and C-4 of quinolinone), 134.29, 134.05, 133.78, 132.88, 132.32, 132.10, 131.11, 129.17, 128.99, 128.84, 128.74, 128.23 128.14, 128.00, 127.89, 126.39, 125.80, 125.62, 124.03, 123.62, 123.15, 122.86, 121.92, 121.79, 121.62, 120.07, 119.86, 117.15, 115.78, 115.58, 114.91, 113.85, 110.90, 110.29, 110.18 (C- aromatic and C-3 of quinolinone of six isomers), 57.04, 56.79, 56.75 (OCH_3 of three isomers), 39.88, 39.26 (CH_2 of 4-dihydroquinolinone of two isomers) ppm. MS: m/z (%) = 374 (M^++2, 13.82), 373 (M^++1, 12.29), 3.72 (M^+, 16.30), 320 (1.29), 319 (1.55), 309 (1.75), 308 (1.81), 307 (2.01), 305 (1.62), 298 (1.82), 297 (2.03), 296 (3.12), 295 (7.92), 294 (15.48), 293 (4.48), 292 (1.48), 271 (1.73), 270 (1.36), 269 (1.34), 268 (1.40), 267 (1.16), 255 (1.27), 243 (1.56), 242 (2.86), 241 (3.54), 240 (1.79), 231 (6.96), 230 (17.58), 229 (11.33), 228 (18.71), 227 (12.23), 224 (1.84), 223 (1.87), 222 (1.85), 216 (3.53), 215 (5.42), 214 (6.26), 213 (4.29), 212 (5.16), 201 (7.33), 200 (14.38), 199

(9.79), 198 (8.16), 197 (6.07), 188 (4.52), 187 (3.22), 186 (4.11), 185 (6.84), 184 (5.18), 183 (3.76), 171 (2.74), 170 (3.12), 169 (3.02), 159 (1.89), 158 (2.52), 157 (1.73), 155 (1.72), 150 (4.52), 148 (1.62), 143 (2.27), 142 (1.42), 135 (2.40), 134 (1.43), 133 (1.46), 132 (1.21), 122 (2.10), 121 (3.75), 106 (14.42), 105 (100), 104 (12.54) , 103 (7.20), 92(3.82), 91 (4.42), 90 (2.02), 79 (6.54), 78 (6.56), 77 (82.91), 76 (17.52), 65 (3.44), 63 (14.63), 62 (7.44), 51 (13.40). Anal. calcd. for $C_{17}H_{13}BrN_2O_3$ (M.wt = 372): C, 54.84; H, 3.49; N, 7.53. Found: C, 54.54; H, 3.23; N, 7.27.

*Synthesis of N-(8-methoxy 6-bromo-2-acetoxyquinolin-3-yl) benzamide (**10**)*

A solution of compound **9** (0.01 mol) in acetic anhydride (20 ml) was refluxed for 4 h. After the reaction was completed, the mixture was poured into water and kept for 24 h. The obtained solid product was separated by filtration, washed with H_2O, and dried. The crude product was subsequently recrystallized from ethanol to give compound **10** a pale-yellow solid, yield 61%, m.p. 158 °C. IR (KBr) ν_{max}: 3222 (NH), 1742, 1679 (C=O), 1631 (C=N), 1605, 1591 (C=C), 1121, 1083, 1021 (C-O) cm^{-1}. ^1H NMR (DMSO-d6) δ: 2.40 (s, 3H, $COCH_3$), 3.83, 3.93, 3.95 (s, 3H, OCH_3 of three isomers), 7.05 (s, 1H, Ar-H), 7.17, 7.19 (d, 1H, J = 8.00 Hz, Ar-H), 7.38-7.39 (d, 1H, J = 4.00 Hz, Ar-H), 7.49-7.50 (d, 1H, J = 4.00 Hz, Ar-H), 7. 55-7.70 (m, 3H, Ar-H, four isomers), 7.75- 7.77 (d, 1H, J = 8.00 Hz, Ar-H), 7.96-7.98 (d, 1H, J = 8.00 Hz, Ar-H), 8.09- 8.10 (d, 1H, J = 4.00 Hz, Ar-H), 8.56, 8.57, 8.86, 8.95 (s, 1H, H-4 of quinoline for four isomers), 9.69, 9.73, 977 (s, 1H, NHCO of three isomers) ppm. ^{13}C NMR (DMSO-d6) δ: 168.72, 166.67, 166.42, 165.12, 157.55 (C=O and N=C-O), 152.45, 147.66 (C-O and C-4 of quinoline), 136.30, 134.73, 133.79, 132.93, 132.86, 129.97, 129.16, 128.82, 128.76, 128.68, 128.17, 128.12, 126.36, 125.79, 125.45, 125.31, 125.2, 123.59, 121.92, 121.79, 120.26, 119.41, 118.51, 117.14, 115.10, 114.93, 110.63 (C-aromatic and C-3 of quinoline of four isomers), 57.23, 57.07, 56.79 (OCH_3 of three isomers), 20.66 ($COCH_3$) ppm. MS: m/z (%) = 414 (M^+, unstable), 374 (2.78), 375 (5.56), 372 (5.56), 292 (1.16), 280 (1.93), 279 (1.89), 278 (1.36), 277 (1.60), 276 (2.10), 213 (1.44), 199 (2.10), 198 (3.52), 197 (1.78), 196 (2.71), 182 (1.16), 170 (1.06), 168 (1.25), 157 (1.17), 155 (1.03), 141 (1.91), 106 (7.59), 105 (100), 104 (11.66), 103 (2.79), 102 (1.74), 89 (1.30), 82 (1.09), 80 (1.44), 77 (29.75), 76 (4.12), 75 (1.52), 63 (2.11), 62 (2.74), 51 (12.03). Anal calcd. for $C_{19}H_{15}BrN_2O_4$ (M.wt = 414): C, 55.07; H, 3.62; N, 6.76. Found: C, 54.83; H, 3.33; N, 6.44.

Biology methods

In vitro cytotoxic *liver cancer cells*

The cytotoxicity of the N-(substituted coumarin-3-yl and/or substituted azacoumarin-3-yl) benzamides (**8a**, **b**, **c,** and **9**, **10**) was studied by MTT-method. Before treating with **8**-**10**, the cells were positioned in a 96-well plate (104 cells per well) for 24 h to permit the cell to be fixed into the well's wall. Various concentrations with 6 wells of each from **8**-**10** components were operated by the cell monolayer throughout the test. Monolayer cells were hatched with the compounds for 48 h at 37 °C and a 5% of CO_2-brooder. The cells were prepared and cleaned before adding MTT-yellow. The color solution was to each well and hatched for 24 h until formazans with purple sediment be seemed. An ELISA plate reader was used to detect the absorbance at 570 nm. The relationship between the surviving fraction and drug concentrations was obtained by plotting to obtain the survival curve of each cancer cell line for the exact compound [31].

Cell cycle analysis

HepG2 cells (2 x 105/well) were cultured for 24 h with the tested compound **8a** at its IC_{50} value. After treatment, the cells were twice-cleaned by 3 mL of ice-cold D-PB5 buffer, centrifugated,

and fixed in ice-cold 70% (v/v) EtOH for 30 min at 4°C before washing with PB5 for another 30 min at 37 °C. The cells were then amassed by centrifugation for 5 min at 2000 rpm and then tainted with propidium iodide buffer (PI). The DNA content was measured by BD FACSCalibur Flow Cytometer-The tests were duplicated [32].

Annexin V-FITC/PI method

The flow cytometric assay with Annexin V-Smearing was used to detect the apoptosis by the Bio-Vision Annexin V-FITC apoptosis uncovering Kit (Cat No. K 10l-25). The IC_{50} concentration of the tested compound **8a** was used for HepG2 cells (2 x 10^5/well). The cells were gathered using trypsin, twice cleaned, with PB5, and re-hanged in 500 L of uniting buffer. After that, cells were dark-incubated for 45 min at RT with 5 mL of Annexin V-FITC and 5 mL of PI. The cells were immediately evaluated with a flow cytometer with a FITC-indicator detector [33].

Tubulin polymerization method

The HepG2 cell line was cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (HyClone), 10 µg/n insulin (Sigma), and 1% penicillin-streptomycin. Plate cells volume of 100 µL complete growth medium and 100 µL of tested compound per well in a 96-well plate for 18-24 h before enzyme assay for tubulin. The microtiter plate provided in this has been pre-coated with antibodies specific to TUBB. Standard samples are then added to the appropriate microtiter plate V with a biotin-conjugated antibody specific to TUBB. Next, A_1 conjugated to Horseradish Peroxidase (HRP) is added to the microplate well and incubated. After the TMB substrate solution is added, only those well contain TUBB, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the action of sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 10 nm concentration of TUBB in the samples is then determined by comparing the O.D. of the samples to the standard curve. All experiments were done in duplicate [34, 35].

CONCLUSION

In conclusion, a series of novel N-(substituted)benzamide derivatives containing coumarin and azocoumarin moieties have been designed. Synthesized compounds and their biological activities were evaluated as potent cytotoxic activity. The results showed promising activity of most of the compounds as an inhibitor of both liver cancer (HepG2) with cytotoxicity. Among them, is compound **8a** good inhibitor activity than other compounds. Further, in vitro cell cycle analysis of compound **8a** revealed that HepG2 cells arrest at G1/S phases of the cell cycle outline, and induction of apoptosis at the pre-G1 stage. Compound **8a** antitumor mechanism was linked to its dual inhibitory effects versus tubulin polymerization.

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