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Synthesis, anticoagulant and PIVKA-II induced by new 4-hydroxycoumarin derivatives

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ABSTRACT

The action of the coumarin-type drugs and related compounds is reviewed to their VKOR antagonistic effects. In our study, twenty 3-pyridinyl, pyrimidinyl and pyrazolyl-4-hydroxycoumarin derivatives were synthesized. A comparative in vivo (CT, PT determination) and in vitro (measurement of PIVKA-II levels) anticoagulant study with respect to warfarin showed that the synthesized compounds have different anticoagulant activities, the most prospective compounds were the 3-pyrazolyl-4-hydroxycoumarin derivatives.

1. Introduction

4-Hydroxycoumarin derivatives as warfarin, acenocoumarol and phenprocoumon have been the mainstay of oral anticoagulation therapy for 20 years,[1] their interest as anticoagulant[2–4] and rodenticides[5–8] has been documented in several reports.

Coumarin anticoagulants are considered as antivitamin K. Vitamin K is an essential cofactor for γ-carboxylation of key glutamic acid residues in blood clotting proteins by vitamin K-dependent carboxylase. During catalysis, vitamin K is converted from the active form to vitamin K 2,3-epoxide, which must be recycled to the active form by vitamin K epoxide reductase (VKOR) to maintain the coagulation cycle.[9,10] 4-Hydroxycoumarins antagonize VKOR, preventing vitamin K recycling and resulting in an accumulation of abnormal form of coagulation protein called des-γ-carboxyprothrombin (DCP) or proteins induced by vitamin K antagonism (PIVKA-II)[11] (Fig. 1).

PIVKA-II has been identified as a marker of hepatocellular carcinoma in humans. An enzyme-linked immunoassay (ELISA) containing monoclonal antibody that specifically detected uncarboxylated prothrombin[12] was developed to diagnose the hepatocellular carcinoma and is also used to clinically monitor the effectiveness of anticoagulant therapy.[13] PIVKA-II ELISA has also been successfully used to detected uncarboxylated prothrombin in culture supernatant of primary human liver cells.[14]

Several reports have provided evidence that human hepatoma cell line, HepG2, secretes PIVKA-II in response to warfarin exposure.[15,16] It was reported in a previous study the development of a detection system for anticoagulation coumarins[17] using ELISA assay to detect PIVKA-II produced by HepG2 exposed to several coumarin compounds.

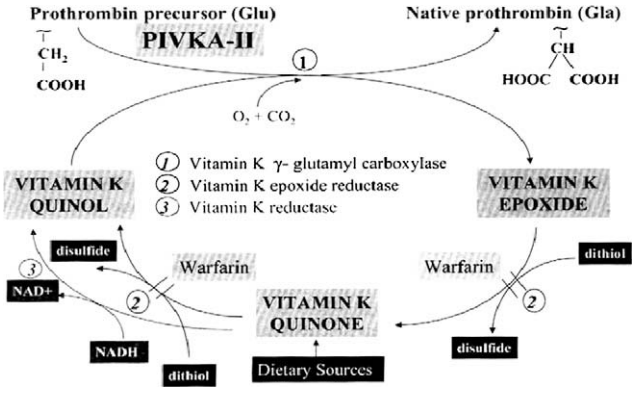

Figure 1. Coumarin anticoagulants antagonizing VKOR.

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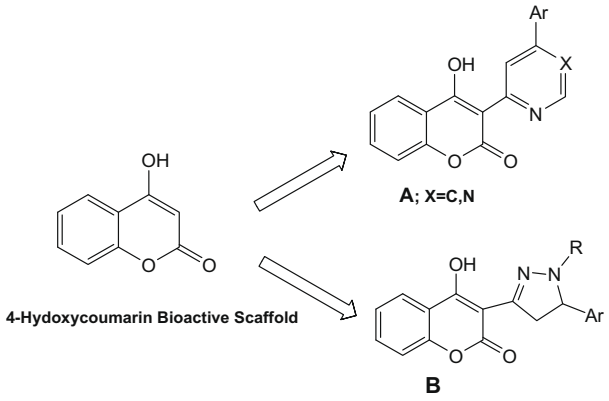
On the other hand, Bhatia et al.,[18] have studied the anticoagulant effects of some pyridyl chromene-2-one derivatives. In addition, condensed coumarinopyrimidines were considered useful leads for the development of new gastric sparing antiplatelet drug with combined anti ADP-TXA_2 activities.[19]

The main objective of our present work depends on the hybridization of 4-hydroxycoumarin unit as anticoagulant scaffold and some azaheterocyclic rings reported to have antithrombotic activity as pyridine[20,21] pyrimidine[19] and pyrazole[22–24] in order to prepare some new molecules (models A and B) to study their in vivo and in vitro anticoagulant activity.



2. Results and discussion

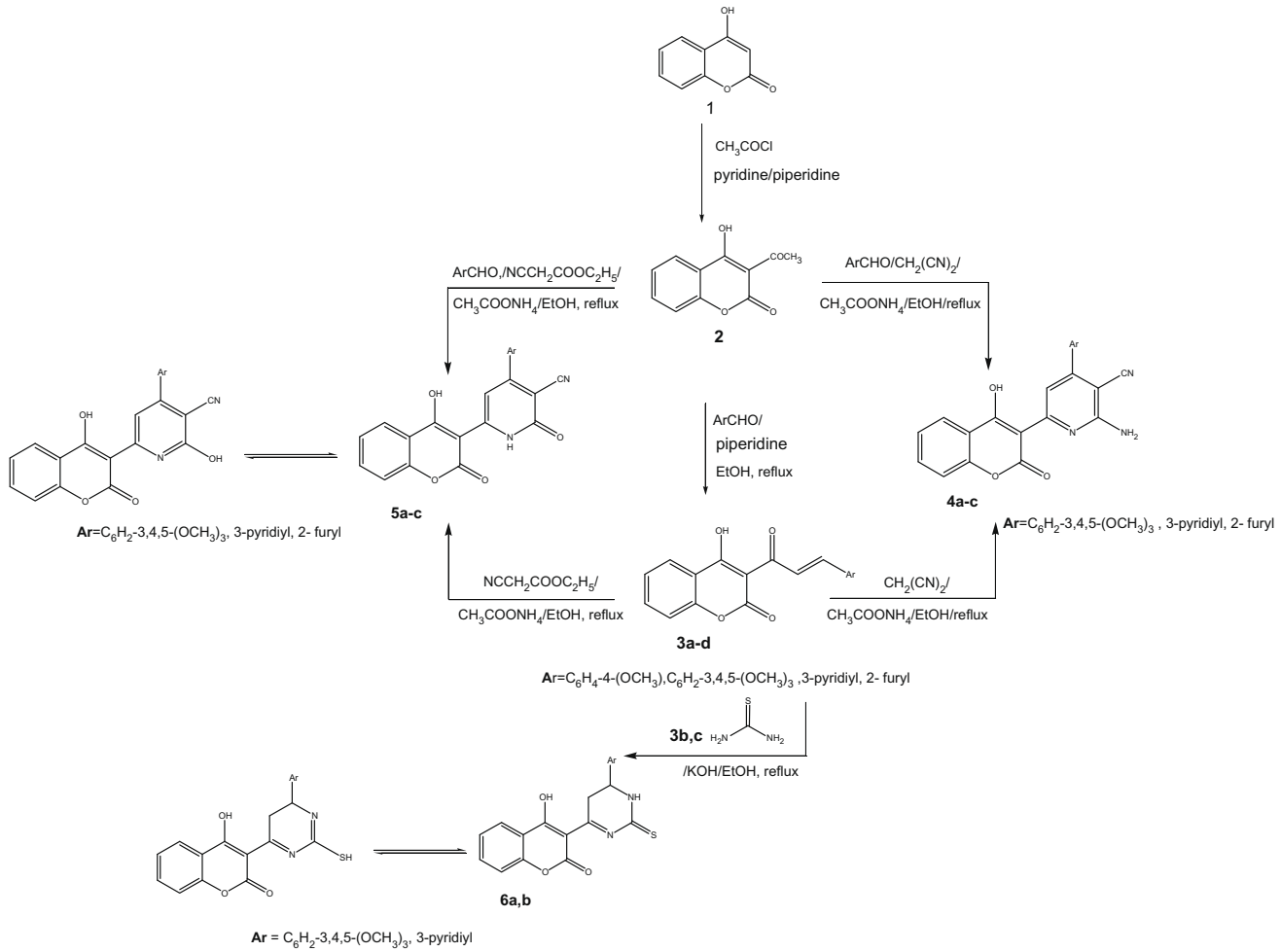
2.1. Chemistry

We described here a convenient approach to the preparation of 4-aryl-2-amino-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-carbonitriles **4a**–**4c** and 4-aryl-2-oxo-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-carbonitriles **5a**–**5c**, 3-(6-aryl-1,2,5,6-tetrahydro-2-thioxo pyrimidin-4-yl)-4-hydroxy-2*H*-chromen-2-one **6a**, **6b** and pyrazol-3-yl-4-hydroxycoumarin derivatives **7a**–**7c**, **8a**, **8b**, **9a**, **9b**, **10a**, **10b** and **11a**–**11c**.

The key reactions involved the intermediate formation of chalcone compounds **3a**–**3d**, a facile synthesis these synthesized α,β-unsaturated ketones involves condensation of 3-acetyl-4-hydroxycoumarin **2**[25] with the appropriate aldehydes; 4-methoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, 3-pyridincarboxaldehyde and 2-furfural in alcohol and in the presence of piperidine.

4-Aryl-2-amino-6-(4-hydroxycoumarin-3yl)-pyridin-3-carbonitriles **4a**–**4c** were prepared by direct reaction of 3-acetyl-4-hydroxycoumarin **2** with the appropriate aldehydes in the presence of malononitrile and ammonium acetate in one step, that is, one pot reaction (method A) (Scheme 1) or by cyclization of the corresponding chalcones with malononitrile and ammonium acetate (method B) (Scheme 1).

One the other hand when the above reactions were carried out in the presence of ethylcyanoacetate instead of malononitrile, 4-aryl-1,2-dihydro-6-(4-hydroxycoumarin-3yl)-2-pyridin-3-carbonitriles **5a**–**5c** were obtained (Scheme 1).



Scheme 1.

The target pyrimidin-2-thiones **6a**, **6b** were obtained in a good yield by cyclocondensation of the α,β-unsaturated ketones **3b**, **3c** with thiourea in 5% ethanolic potassium hydroxide solution (Scheme 1).

Heterocyclization of the appropriate hydrazines with electrophilic species such as α,β-unsaturated ketones afforded the corresponding pyrazolines; cyclocondensation of the key intermediates **3a**–**3c** with hydrazine hydrate in ethyl alcohol gave pyrazolines **7a**–**7c**. Similarly, *N*-acetylpyrazolines **8a**, **8b** were obtained by refluxing the key chalcones **3b**, **3c** with hydrazine hydrate in glacial acetic acid. *N*-Phenylpyrazolines **9a**, **9b** were also prepared from the proper chalcones **3b**, **3c** and phenylhydrazine (Scheme 2).

Moreover, the target compounds **10a**, **10b** were prepared by refluxing a mixture of chalcones **3a**, **3c** and thiosemicarbazide in ethyl alcohol in the presence of glacial acetic acid (Scheme 2). The target thioureas **11a**–**11c** were prepared by the reaction of pyrazolines **7a**–**7c** with phenyl isothiocyanate in dry acetone at room temperature (Scheme 2). The proposed structures of all new prepared compounds were confirmed by elemental analysis, IR, ^{1}H NMR, ^{13}C NMR and mass spectra (Section 3).

2.2. Pharmacological screening

2.2.1. In vitro anticoagulant activity

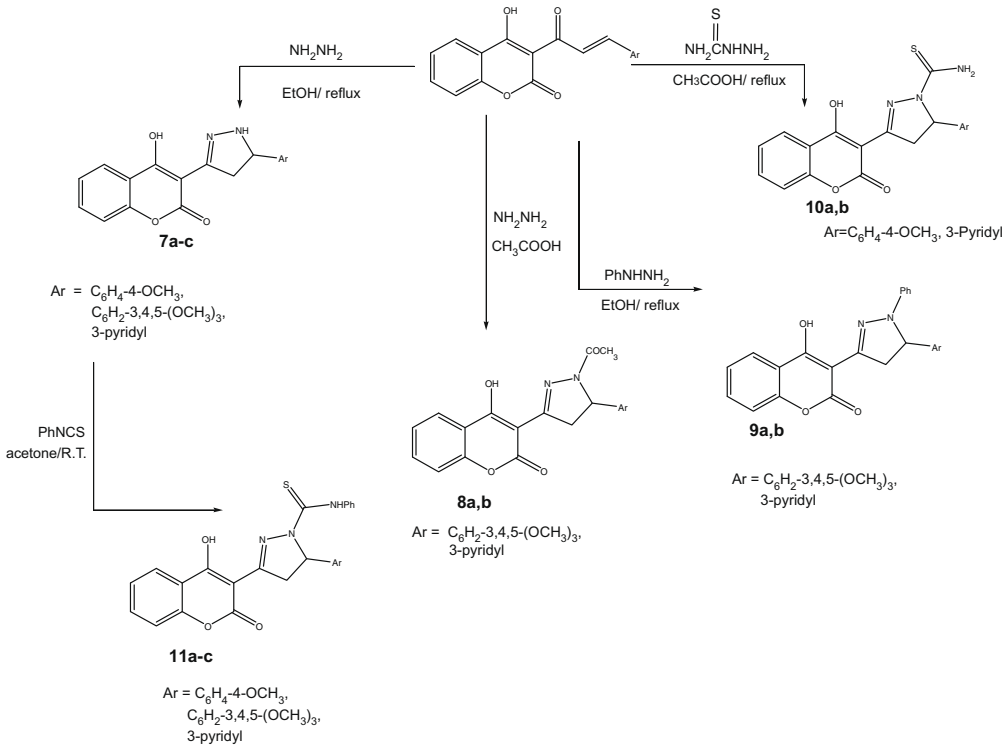
In this study, a generic biological screening assay was designed to detect the accumulation of under-carboxylated prothrombin or proteins induced by vitamin K antagonism (PIVKA-II). A combined cell culture/ELISA assay was optimized to measure PIVKA-II production by the human hepatoma HepG2 cell line cultured in the presence of anticoagulant coumarins.

On each ELISA plate, four reference standards (2, 50, 120 and 200 ng/ml) provided with the commercially-available kit were tested at the same dilutions as the new chemical entities. PIVKA-II production by HepG2 cells is considered negative if assay values are below the 2 ng/ml reference standard (0.158 nm). A value of 200 ng/ml represents a high positive result (1.982 nm). Under the same experimental conditions, exposure of HepG2 cells to 0.03, 0.3, 3, 10 and 30 μM warfarin resulted in an approximate concentration-dependent increase in PIVKA-II in cell supernatants (0.577, 1.1135, 1.137, 2.282 and 2.342 nm, respectively). In contrast, a value of less than 2 ng/ml PIVKA-II (0.063 nm) was detected following exposure to 0.003 μM warfarin.

The anticoagulant activity of the synthesized coumarin derivatives (**4c**, **5a**–**5c**, **6a**, **6b**, **7a**–**7c**, **8b**, **9a**, **9b**, **10a**, **10b** and **11a**–**11c**) was determined at different concentrations according to their solubility using an ELISA assay that detects proteins induced by vitamin K antagonism-II (PIVKA-II) in HepG2 cells, compounds **4a**, **4b** and **8a** were not tested due to their low solubility (Table 1).

PIVKA-II production was clearly detected in supernatants after treatment with compounds **4c** (30 μM), **5b** (10 μM), **6a** (30 μM), **7a** (30 μM), **10a** (30 μM), **10b** (30 μM) and **11c** (30 μM) (Table 1). These tested compounds while active, were less potent than warfarin at 0.003 μM (0.577 nm). The other tested compounds gave values below the 2 ng/ml reference standard and thus are considered void of significant anticoagulant activity.

Although the relation between the structure of the tested coumarin-C3-linked azaheterocycles and their in vitro anticoagulant activity results is not very clear, the following points can still be concluded. The pyrazolyl coumarin derivatives **11c** (R.P. 19.4%), **7a** (R.P. 17.3%) and **10a** (R.P. 18.1%) were proved to be the most active of the tested compounds. The relative anticoagulant potency of compound **11c** was decreased to 11.2%, 11.45% and 8.7% when the phenylcarbothioamide group in position 1 of the pyrazolyl functionality was replaced with carbothioamide group (compound **10b**), acetyl function (compound **8b**) or phenyl nucleus (compound **9b**), respectively. Pyrazolyl coumarins that contained 3,4,5-trimethoxyphenyl substituent in position 5 of the pyrazolyl moiety were inactive; for example, the activity of compounds **11c** and **7a** was lost by substituting the C-5 pyridine function or C-5 4-methoxyphenyl group on the pyrazole ring, respectively, with 3,4,5-trime-



Scheme 2.

Table 1
Screening measurement of proteins induced by vitamin K antagonism-II (PIVKA-II) as indicator of anticoagulation activity

Tested compounds	ABS (490 nm) ± SD	PIVKA-II
4c-30 μM	0.23425 ± 0.003536	Yes
5a-30 μM	0.057 ± 0.003	No
5b-10 μM	0.1865 ± 0.008	Weak
5c-30 μM	0.207 ± 0.0045	Weak
6a-30 μM	0.30725 ± 0.00495	Yes
6b-30 μM	0.20625 ± 0.0047	Weak
7a-30 μM	0.40625 ± 0.012728	Yes
7b-30 μM	0.071 ± 0.001	No
7c-30 μM	0.07 ± 0.007	No
8b-3.3 μM	0.205 ± 0.00432	Weak
9a-3.3 μM	0.059 ± 0.008	No
9b-30 μM	0.2025 ± 0.0058	Weak
10a-30 μM	0.4235 ± 0.008991	Yes
10b-30 μM	0.26175 ± 0.0047	Yes
11a-30 μM	0.0535 ± 0.006	No
11b-3.3 μM	0.05 ± 0.000	No
11c-30 μM	0.4535 ± 0.01879	Yes

thoxyphenyl group, compounds **11b** and **7b**, respectively (Tables 1 and 2).

Concerning the coumarinyl pyridines derivatives, compound **5b** (R.P. 10%) and **4c** (R.P. 10%) showed anticoagulant activity higher than that of the reference standard at 2 ng/ml (0.158 nm). replacement of the 4-pyridinyl substituent of the pyridine functionality in compound **5b** with furan nucleus or 3,4,5-trimethoxyphenyl group resulted in a decrease (compound **5c**) or complete loss (compound **5a**) of the activity.

The coumarinopyrimidine derivatives **6a** (R.P. 13%) and **6b** (R.P. 8%) showed anticoagulant activity value in PIVKA-II assay comparable to that of the pyridinyl coumarin **5b** and **4c**, respectively (Tables 1 and 2).

2.2.2. In vivo anticoagulant screening

This study was conducted to evaluate the in vivo effects of the new synthesized compounds **4a**, **5a**, **6a**, **7c**, **8b**, **9b**, **10b** and **11c** on blood coagulation time (CT) and prothrombin time (PT) in mice, warfarin, the reference compound, was tested under the same conditions. From the anticoagulant values obtained in Table 3, modest to high activity over warfarin was observed for the pyrazolyl coumarin derivatives **7c**, **8b**, **9b**, **10c** and **11c**. The effect of compound **7c** (CT; 17.67 s) increased by introduction of acetyl group or phenyl nucleus at position 1 of the pyrazolyl functionality, compounds **8b** (CT; 20.83 s) and **9b** (CT; 22.50 s), respectively.

Additionally, compounds **8b** (PT; 3.373 min), **10b** (PT; 4.017 min) and **11c** (PT; 3.505 min) with acetyl, carbothioamide and phenylcarbothioamide, respectively, in the pyrazolyl moiety showed pronounced prolongation of PT with anticoagulant values similar to that of warfarin (PT; 4.445 min) (Table3).

On the other hand, the tested pyridyl and pyrimidinyl coumarin derivatives **5a** and **6a** had relatively moderate in vivo clotting activity with CT values 15.67 and 15.83 s, both compounds and **4a** showed no effect on PT.

LD50 (po) of the most active compound **9b** (CT; 22.50 ± 0.96) was determined as 1790 mg/kg. (Table 4)[26](cf. LD50 (po) of warfarin 760 mg/kg).

2.2.3. Conclusion

In conclusion, a series of 4-hydroxycoumarins incorporating pyridine, pyrimidine and pyrazole nucleus linked at C-3 has been prepared as potential anticoagulant agents. These azaheterocycles were selected because they have historically provided improved in vitro and/or in vivo antithrombotic potency. We have identified that the hybrid pyrazolyl coumarins, **11c**, **10b** and **7a**, are the most active tested compounds. The ambiguity between in vitro (low) and in vivo (high) anticoagulant values may be attributed to their in vivo dual mechanism of action of coumarin pharmacophore and pyrazole pharmacophore group. The in vitro assay abundant represents a single step in anticoagulant cascade. However, in vivo assay

Table 2
Relative potency of positive and Weak anticoagulants

Test compounds	Concd	Avg. Abs. SD	Relative potency% in relation to warfarin (R.P. %)
Reference	2 ng/ml	0.158	
4c	30 μM	0.23425 ± 0.003536	**10%**
	10 μM	0.169 ± 0.00495	7.4%
5b	10 μM	0.24875 ± 0.009	**10%**
	3.33 μM	0.1355 ± 0.004243	—*
5c	30 μM	0.207 ± 0.0045	**8.8%**
	10 μM	0.2055 ± 0.0034	**9%**
6a	30 μM	0.30725 ± 0.00495	**13%**
	10 μM	0.119 ± 0.000707	5.2%
6b	30 μM	0.20625 ± 0.0047	**8%**
	10 μM	0.117 ± 0.006364	—*
7a	30 μM	0.40625 ± 0.012728	**17.3%**
	10 μM	0.4055 ± 0.018385	**17.76%**
8b	3.3 μM	0.205 ± 0.00432	**11.45%**
	1.1 μM	0.0075 ± 0.001	—*
9b	30 μM	0.2025 ± 0.0058	**8.7%**
	10 μM	0.192 ± 0.00428	**8.4%**
10a	30 μM	0.4235 ± 0.008991	**18.1%**
	10 μM	0.3135 ± 0.00495	**13.7%**
10b	30 μM	0.26175 ± 0.0047	**11.2%**
	10 μM	0.207 ± 0.005657	**9.1%**
11c	30 μM	0.4535 ± 0.01879	**19.4%**
	10 μM	0.441 ± 0.007778	**19.3%**

* Compound showed no activity.

Table 3
Effect of tested compounds and warfarin (150 mg/kg, po) on clotting time (s) and prothrombine time (min) in mice after three days of daily treatments (mean ± SE, n = 6/group)

Tested compounds 150 mg/kg	CT/s	PT/min
Control	8.17 ± 0.60	1.283 ± 0.069
4a	9.50 ± 0.62	0.193 ± 0.036
5a	15.67 ± 1.05	0.192 ± 0.010
6a	15.83 ± 0.95	0.173 ± 0.007
7c	17.67 ± 0.80	0.530 ± 0.020
8b	20.83 ± 0.79	3.373 ± 0.257
9b	22.50 ± 0.96	0.827 ± 0.141
10b	16.67 ± 0.71	4.017 ± 0.292
11c	12.17 ± 0.79	3.505 ± 0.226
Warfarin	18.17 ± 0.79	4.445 ± 0.314
LSD*	2.26	0.5

* LSD: least significant difference.

Table 4
LD50 in male mice after oral administration of **9b**

Group No.	Oral doses (mg/kg. b. wt.)	No. of dead animals	Dose difference	Mean[a]	Product[b]
1	1200	—	200	—	
2	1400	2	200	1	200
3	1600	4	200	3	600
4	1800	5	200	4.5	900
5	2000	6	200	5.5	1100
6	2200	8	200	7.5	1500
7	2400	10	200	9	1800
Total					**6100**

No of animals/group = 10 mice.
LD50 = 2400 − (6100/10) = 1790 mg/kg. b.wt.
[a] Interval mean of the number of dead animals (mice).
[b] Product of the interval mean and the dose difference.

represents the total anticoagulant activity, so the in vitro data do not represent the real state of the anticoagulation activity. We hope that the efficient synthesis approach disclosed herein can lead to the rapid output of a series of pyrazolylcoumarins for evaluation and anticoagulant mechanism studies.

3. Experimental

3.1. Synthesis

Melting points were determined on Electrothermal IA 9000 apparatus and were uncorrected. Elemental microanalysis was performed on Elementar, Vario EL, at the microanalytical center, National Research Center. The infrared (IR) spectra were recorded on Nexus 670 FT-IR FT-Raman spectrometer as potassium bromide discs, at National Research Center. The proton nuclear magnetic resonance (^{1}H NMR) spectra were determined on Varian mercury 300 MHz spectrometer, using tetramethylsilane (TMS) as an internal standard, at Faculty of Science—Cairo University and National Research Center. ^{13}C NMR was carried out using 1D–^{13}C NMR Bruker Jeol-EX 125 MHz spectrometer using TMS as an internal standard. The mass spectra (MS) were performed on Jeol JMS-AX500 mass spectrometer at National Research Center. The reactions were followed by TLC (silica gel, aluminum sheets 60 F 254, Merk) using benzene/ethyl acetate (8:2 v/v) as eluent and sprayed with iodine–potassium iodide reagent.

3.1.1. Preparation of 1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-3-aryl-2-propen-1-ones (3a–3d)

A solution of 3-acetyl-4-hydroxycoumarin[25] (1 g, 5 mmol) in ethyl alcohol (10 ml) and the selected aromatic aldehyde, namely, 4-methoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, 3-pyridincarboxaldehyde or 2-furfural (5 mmol) in the presence of piperidine (1 ml) was refluxed for 10–12 h. The solution was cooled and water was added to precipitate the desired chalcone compounds.

3.1.1.1. 1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)-3-(4-methoxyphenyl)-2-propen-1-one (3a)[27]. Mol. for.: $C_{19}H_{14}O_5$, M.wt. 322.31, yield 91%, mp 148–150 °C.

3.1.1.2. 1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-one (3b)[27]. Mol. for.: $C_{21}H_{18}O_7$, M.wt. 382.36, yield 93%, mp 157–159 °C.

3.1.1.3. 1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)-3-(pyridin-3-yl)-2-propen-1-one (3c). Mol. for.: $C_{17}H_{11}NO_4$, M.wt. 293.27, yield 88%, mp 190–192 °C; Anal. Calcd: C, 69.62; H, 3.78; N, 4.78. Found: C, 69.50; H, 3.63; N, 4.58; IR (KBr, cm^{-1}): 3339 (OH, br s), 3099 (–CH aliphatic stretching), 1677 (C=O, α-pyrone), 1648 (C=O), 1607 (C=N), 1574 (C=C); ^{1}H NMR (DMSO-d_6, δ, ppm): 7.03, 7.08 (2H, d, CH=CH), 7.13–8.66 (8H, m, Ar-H and pyridine protons), 12.92 (1H, s, OH, D_2O exchangeable). ^{13}C NMR (DMSO, δ, ppm):98.92, 117.43, 122.52, 123.74, 125.11, 127.01, 128.42, 129.63, 132.92, 133.43, 148.92, 150.10, 150.32, 152.71, 160.03, 179.75, 183.84; MS; *m*/*z* (R.A. %): (M^++1) 292 (100%).

3.1.1.4. 1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)-3-(furan-2-yl)-2-propen-1-one (3d)[28]. Mol. for.: $C_{16}H_{10}O_5$, M.wt. 282.25, yield 65%, mp 204–205 °C.

3.1.2. Preparation of 4-aryl-2-amino-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-carbonitriles (4a–4c)

General procedure. Method (A): To a mixture of 3-acetyl-4-hydroxycoumarin (1 g, 5 mmol) and the appropriate aldehyde, namely; 3,4,5-trimethoxybenzaldehyde, 3-pyridincarboxaldehyde or 2-furfural (5 mmol) in ethyl alcohol (20 ml), malononitrile (0.33 g, 5 mmol) and ammonium acetate (0.75 g, 10 mmol) were added. The reaction mixture was refluxed for 7–9 h. The obtained solid was filtered off, washed with absolute ethyl alcohol and recrystallized from methyl alcohol to give the desired compounds.

Method (B): An ethanolic mixture of the selected chalcones **3b**–**3d** (5 mmol), and malononitrile (0.33 g, 5 mmol) in the presence of ammonium acetate (0.75 g, 10 mmol) was refluxed for 5–6 h, after cooling, the obtained solid was filtered off, washed with ethyl alcohol and recrystallized from methyl alcohol to give the title compounds.

3.1.2.1. 2-Amino-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-4-(3,4, 5-trimethoxy phenyl)pyridine-3-carbonitrile (4a)[27]. Mol. for.: $C_{24}H_{19}N_3O_6$, M.wt. 445.42, yield 90% (method A), 83% (method B), mp >300 °C.

3.1.2.2. 2-Amino-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-4-(pyridine-3-yl) pyridin-3-carbonitrile (4b). Mol. for.: $C_{20}H_{12}N_4O_3$, M.wt. 356.33, yield 88% (method A), 78% (method B), mp >300 °C; Anal. Calcd: C, 67.41; H, 3.39; N, 15.72. Found: C, 67.21; H, 3.22; N, 15.61. IR (KBr, cm^{-1}): 3392 (OH, br s), 3118, 3099 (NH_2), 2210 (C≡N), 1722 (C=O, α-pyrone), 1653 (C=N). ^{1}H NMR (DMSO-d_6, δ, ppm): 4.31 (2H, s, NH_2, D_2O exchangeable), 7.33–8.86 (9H, m, Ar-H and pyridine protons), 8.92 (1H, s, OH, D_2O exchangeable). ^{13}C NMR (DMSO, δ, ppm): 88.62, 98.06, 104.28, 117.40, 117.95, 121.95, 124.40, 125.95, 127.89, 128.48, 133.62, 134.92, 148.40, 149.92, 150.62, 152.31, 159.95, 162.17, 162.40, 166.73; MS; *m*/*z* (R.A. %): M^+ 356 (10%), 340 (54%), 339 (48%), 338 (54%), 311 (100%).

3.1.2.3. 2-Amino-4-(furan-2-yl)-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-carbonitrile (4c). Mol. for.: $C_{19}H_{11}N_3O_4$, M.wt. 345.31, yield 74% (method A), 69% (method B), mp >300 °C; Anal. Calcd: C, 66.09; H, 3.21; N, 12.17. Found: C, 65.96; H, 3.17; N, 12.15. IR (KBr, cm^{-1}): 3316 (OH, br s), 3208, 3057 (NH_2), 2206 (C≡N), 1680 (C=O, α-pyrone), 1643 (C=N). ^{1}H NMR (DMSO-d_6, δ, ppm): 4.01 (2H, s, NH_2, D_2O exchangeable), 6.35–7.41 (3H, m, furyl protons), 7.01–7.65 (4H, m, AR-H), 7.5 (1H, s, CH-pyridine proton), 13.92 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 88.62, 98.06, 104.28, 105.3, 107.52, 117.40, 117.95, 121.95, 125.95, 127.89, 128.48, 143.29, 150.62, 154.30, 152.31, 159.95, 162.17, 162.40, 166.73; MS; *m*/*z* (R.A. %): (M^++1) 345 (6%), 239 (10%), 202 (31%), 144 (12%), 92 (15%), 77 (70%), 62 (100%), 45 (64%).

3.1.3. Preparation of 4-aryl-1,2-dihydro-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-oxopyridin-3-carbonitriles (5a–5c)

General procedure. Method (A): To a mixture of compound **2** (1 g, 5 mmol) and the appropriate aldehyde, namely; 3,4,5-trimethoxybenzaldehyde, 3-pyridincarboxaldehyde or 2-furfural (5 mmol) in ethyl alcohol (20 ml), ethyl cyanoacetate (0.57 ml, 5 mmol) and ammonium acetate (0.75 g, 10 mmol) were added. The reaction mixture was refluxed for 7–9 h. The obtained solid was filtered off, washed with absolute ethyl alcohol and recrystallized from methanol to give the desired compounds.

Method (B): An ethanolic mixture of chalcones **3b**–**3d** (5 mmol), and ethyl cyanoacetate (0.57 ml, 5 mmol) in the presence of ammonium acetate (0.7 g, 10 mmol) was refluxed for 5–6 h, after cooling, the obtained solid was filtered off, washed with ethyl alcohol and recrystallized from methyl alcohol to give the title compounds.

3.1.3.1. 1, 2-Dihydro-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-4-(3,4,5-trimethoxyphenyl)-2-oxopyridin-3-carbonitrile (5a). Mol. for.: $C_{24}H_{18}N_2O_7$, M.wt. 446.41, yield 75% (method A), 63% (method

B), mp 223–225 °C; Anal. Calcd: C, 64.57; H, 4.06; N, 6.28. Found: C, 64.34; H, 3.99; N, 6.09. IR (KBr, cm^{-1}): 3264 (OH, br s), 3043 (NH), 2364 (C≡N), 1697 (C=O, α-pyrone), 1612 (C=O). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.82 (9H, s, CH3), 5.91 (1H, s, oxopyridine proton), 6.83–7.56 (6H, m, AR-H), 8.01 (1H, s, NH, D_2O exchangeable), 11.81 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 56.22, 56.22, 56.75, 95.22, 99.19, 104.19, 104.19, 115.43, 115.71, 117.75, 121.75, 125.75, 126.80, 127.19, 128.64, 138.19, 138.63, 150.23, 150.70, 150.70, 158.53, 159.54, 161.86, 166, 92; MS; *m*/*z* (R.A. %): (M^++1) 447 (12%), 379 (18%), 281 (25%), 181 (61%), 121 (33%), 78 (83%), 63 (75%), 43 (100%).

3.1.3.2. 1,2-Dihydro-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-oxo-4-(pyridine-3-yl) pyridin-3-carbonitrile (5b). Mol. for.: $C_{20}H_{11}N_3O_4$, M.wt. 357.32, yield 70% (method A), 68% (method B), mp 219–222 °C; Anal. Calcd: C, 67.23; H, 3.10; N, 11.76. Found: C, 67.21; H, 3.02; N, 11.58. IR (KBr, cm^{-1}): 3449 (OH, br s), 3057 (NH), 2739 (C≡N), 1739 (C=O, α-pyrone), 1692 (C=O). ^{1}H NMR (DMSO-d_6, δ, ppm): 5.99 (1H, s, oxopyridine proton), 7.33–8.41 (7H, m, AR-H and pyridinyl protons), 8.50 (1H, s, CH-pyridine proton), 8.61 (1H, s, NH, D_2O exchangeable), 10.71 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 95.22, 99.19, 115.43, 117.75, 121.32, 121.75, 124.15, 125.75, 126.90, 127.19, 133.42, 133.72, 138.19, 149.81, 150.01, 150.25, 158.53, 159.64, 161.86, 166.92; MS; *m*/*z* (R.A. %): (M^++1) 358 (0.9%), 201 (33%), 146 (19%), 120.5 (21%), 61 (24%), 60 (98%), 62 (100%), 43 (84%).

3.1.3.3. 4-(Furan-2-yl)-1,2-dihydro-6-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-oxopyridin-3-carbonitrile (5c). Mol. for.: $C_{19}H_{10}N_2O_5$, M.wt. 346.29, yield 74% (method A), 69% (method B), mp 228–230 °C; Anal. Calcd: C, 65.90; H, 2.91; N, 8.09. Found: C, 65.82; H, 2.78; N, 8.11. IR (KBr, cm^{-1}): 3262 (OH, br s), 3098 (NH), 2354 (C≡N), 1696 (C=O, α-pyrone), 1658 (C=O). ^{1}H NMR (DMSO-d_6, δ, ppm): 5.09 (1H, s, oxopyridine proton), 6.73–8.01 (7H, m, AR-H and furyl protons), 8.63 (1H, s, NH, D_2O exchangeable), 12.71 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 95.22, 99.19, 111.54, 112.97, 115.43, 117.75, 121.32, 121.75, 125.75, 126.90, 127.19, 138.19, 145.91, 149.82, 150.21, 158.53, 159.64, 161.86, 166.42; MS; *m*/*z* (R.A. %): (M^++2) 348 (1%), 322 (19%), 279 (4%), 187 (25%), 163 (85%), 146 (61%).

3.1.4. Preparation of 4–3-(6-aryl-1,2,5,6-tetrahydro-2-thioxopyrimidin-4-yl)-4-hydroxy-2*H*-chromen-2-one derivatives (6a, 6b)

To a mixture of the selected chalcones **3b**, **3c** (1 mmol) and thiourea (0.3 g, 4 mmol) in ethyl alcohol (25 ml), a solution of potassium hydroxide (0.4 g) in water (4 ml) was added and the reaction mixture was refluxed for 10–12 h. The solvent was evaporated under vacuum till dryness and the residue was acidified with dilute hydrochloric acid, the formed solid was filtered off and crystallized from methyl alcohol to give compounds **6a**, **6b**.

3.1.4.1. 3-[1,2,5,6-Tetrahydro-6-(3,4,5-trimethoxyphenyl)-2-thioxopyrimidin-4-yl]-4-hydroxy-2*H*-chromen-2-one (6a). Mol. for.: $C_{22}H_{20}N_2O_6S$, M.wt. 440.47, yield 75%, mp 242–245 °C; Anal. Calcd: C, 59.99; H, 4.58; N, 6.36; S, 7.28. Found: C, 59.68; H, 4.55; N, 6.11; S, 7.20. IR (KBr, cm^{-1}): 3558 (OH, br s), 3174 (NH), 2930 (–CH aliphatic stretching), 1716 (C=O, α-pyrone), 1240 (C=S). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.71 (1H, s, NH, D_2O exchangeable), 3.42 (1H, dd, CH-pyrimidine), 3.98 (9H, s, $3OCH_3$), 4.34 (1H, dd CH-pyrimidine), 4.76 (1H, dd, CH-pyrimidine), 6.62–8.23 (6H, m, Ar-H), 15.31 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 35.02, 53.41 56.23, 56.23, 56.75, 82.21, 104.31, 104.31, 117.53, 121.75, 125.45, 126.80, 128.24, 137.84, 138.21, 150.72, 150, 72, 151.01, 159.43, 165.33, 181.01, 188.06; MS; *m*/*z* (R.A. %):M^+ 440 (28%), 395 (16%), 290 (17%), 244 (36%), 121 (100%).

3.1.4.2. 3-[1,2,5,6-Tetrahydro-6-(pyridin-3-yl)-2-thioxopyrimidin-4-yl]-4-hydroxy-2*H*-chromen-2-one (6b). Mol. for.: $C_{18}H_{13}N_3O_3S$, M.wt. 351.38, yield 80%, mp 235–237 °C; Anal. Calcd: C, 61.53; H, 3.73; N, 11.96; S, 9.13. Found: C, 61.03; H, 3.33; N, 11.77; S, 9.07. IR (KBr, cm^{-1}): 3331 (OH, br s), 3208 (NH), 1714 (C=O, α-pyrone), 1299 (C=S). ^{1}H NMR (DMSO-d_6, δ, ppm): 2.00 (1H, s, NH, D_2O exchangeable), 3.81 (1H, dd, CH-pyrimidine), 4.42 (1H, dd, CH-pyrimidine), 4.75 (1H, dd, CH-pyrimidine), 7.20–8.63 (8H, m, Ar-H and pyridinyl protons), 15.31 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 35.06, 52.31, 81.81, 117.23, 121.65, 123.15, 125.15, 126.56, 128.22, 133.32, 140.84, 147.06, 148.35, 150.02, 159.13, 165.03, 181.05, 186.56; MS; *m*/*z* (R.A. %): (M^++1) 352 (100%), 337 (7%), 290 (47%), 245 (52%), 235 (43%), 213 (31%), 119 (71%), 76 (100%).

3.1.5. Preparation of 3-(5-aryl-4,5-dihydro-1*H*-pyrazol-3-yl)-4-hydroxy-2*H*-chromen-2-one derivatives (7a–7c)

A mixture of the appropriate chalcones **3a**–**3c** (1 mmol) and hydrazine hydrate 99% (2 mmol) in ethyl alcohol (30 ml) was refluxed for 1 h. The reaction mixture was cooled and the formed precipitate was filtered off, washed and recrystallized from methyl alcohol to give **7a**–**7c** compounds.

3.1.5.1. 3-[4,5-Dihydro-5-(4-methoxyphenyl)-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (7a)[27]. Mol. for.: $C_{19}H_{16}N_2O_4$, M.wt. 336.34, yield 81%, mp 196–198 °C.

3.1.5.2. 3-[4,5-Dihydro-5-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (7b)[27]. Mol. for.: $C_{21}H_{20}N_2O_6$, M.wt. 396.39, yield 80%, mp 185–187 °C.

3.1.5.3. 3-[4,5-Dihydro-5-(pyridine-3-yl)-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (7c). Mol. for.: $C_{17}H_{13}N_3O_3$, M.wt. 307.30, yield 79%, mp 233–235 °C; Anal. Calcd: C, 66.44; H, 4.26; N, 13.67. Found: C, 66.13; H, 4.06; N, 13.62. IR (KBr, cm^{-1}): 3430 (OH, br s), 3187 (NH), 1675 (C=O, α-pyrone), 1603 (C=N). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.81 (1H, dd, H-pyrazoline), 4.04 (1H, dd, H-pyrazoline), 5.36 (1H, dd, H-pyrazoline), 6.86 (1H, s,NH), 7.02–8.84 (8H, m, Ar-H and pyridinyl protons), 14.23 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 41.42, 49.23, 88.05, 117.56, 121.65, 123.15, 125.05, 126.56, 128.42, 133.35, 140.24, 147.03, 148.31, 150.22, 156.02 159.13, 165.09; MS; *m*/*z* (R.A. %): (M^++1) 308 (100%).

3.1.6. Preparation of 3-(5-aryl-1-acetyl-4,5-dihydro-1*H*-pyrazol-3-yl)-4-hydroxy-2*H*-chromen-2-ones (8a, 8b)

A mixture of chalcones **3b**, **3c** (1 mmol) and hydrazine hydrate 99% (2 mmol) in glacial acetic acid (30 ml) was refluxed for 30–45 min. The reaction mixture was cooled and diluted with water; the formed precipitate was filtered off, washed and recrystallized from ethyl alcohol to give compounds **8a**, **8b**.

3.1.6.1. 3-[1-Acetyl-4,5-dihydro-5-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (8a)[27]. Mol. for.: $C_{23}H_{22}N_2O_7$, M.wt. 438.43, yield 82%, mp 203–204 °C.

3.1.6.2. 3-[1-Acetyl-4,5-dihydro-5-(pyridin-3-yl)-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (8b). Mol. for.: $C_{19}H_{15}N_3O_4$, M.wt. 349.34, yield 87%, mp 218–220 °C; Anal. Calcd: C, 65.32; H, 4.33; N, 12.03. Found: C, 65.11; H, 4.31; N, 12.24. IR (KBr, cm^{-1}): 3410 (OH, br s), 2941 (–CH aliphatic stretching), 1718 (C=O, α-pyrone), 1663 (C=O), 1617 (C=N). ^{1}H NMR (DMSO-d_6, δ, ppm): 2.21 (3H, s, $COCH_3$), 3.85 (1H, dd, H-pyrazo-

line), 4.64 (1H, dd, H-pyrazoline), 5.56 (1H, dd, H-pyrazoline), 7.08–8.55 (8H, m, Ar-H and pyridinyl protons), 14.93 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 23.08, 38.32, 59.34, 88.21, 117.49, 121.15, 123.35, 125.45, 126.52, 128.41, 133.39, 140.84, 147.02, 148.51, 150.04, 156.12 159.83, 166.19, 168.32; MS; *m*/*z* (R.A. %): (M^++1) 350 (100%).

3.1.7. Preparation of 3-(5-aryl-4,5-dihydro-1-phenyl-1*H*-pyrazol-3-yl)-4-hydroxy-2*H*-chromen-2-one derivatives (9a, 9b)

A mixture of the selected chalcones **3b**, **3c** (1 mmol) and phenyl hydrazine (0.1 ml, 1 mmol) in ethyl alcohol (30 ml) was refluxed for 1 h. The reaction mixture was cooled and the formed precipitate was filtered off, washed and recrystallized from methyl alcohol to give the desired compounds.

3.1.7.1. 3-[4,5-Dihydro-5-(3,4,5-trimethoxyphenyl)-1-phenyl-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (9a)[27].

Mol. for.: $C_{27}H_{24}N_2O_6$, M.wt. 472.49, yield 95%, mp 191–192 °C.

3.1.7.2. 3-[4,5-Dihydro-1-phenyl-5-(pyridin-3-yl)-1*H*-pyrazol-3-yl]-4-hydroxy-2*H*-chromen-2-one (9b).

Mol. for.: $C_{23}H_{17}N_3O_3$, M.wt. 383.4, yield 97%, mp 218–220 °C; Anal. Calcd: C, 72.05; H, 4.47; N, 10.96. Found: C, 72.35; H, 4.39; N, 10.82. IR (KBr, cm^{-1}): 3421 (OH, br s), 1715 (C=O, α-pyrone), 1605 (C=N). ^1H NMR (DMSO-d_6, δ, ppm): 3.83 (1H, dd, H-pyrazoline), 4.24 (1H, dd, H-pyrazoline), 5.36 (1H, dd, H-pyrazoline), 6.98–8.15 (13H, m, Ar-H), 14.03 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 38.35, 53.82, 88.05, 113.46, 113.46 117.27, 117.53, 121.65, 123.45, 125.55, 126.76, 128.42, 129.70, 129.70, 133.45, 140.73, 143.90, 147.42, 148.66, 150.21, 155.72, 159.63, 166.18; MS; *m*/*z* (R.A. %): (M^+) 383 (35%), 381 (23%), 307 (17%), 241 (17%), 183 (22%), 76 (67%), 69 (52%), 63 (100%).

3.1.8. Preparation of 5-aryl-4,5-dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyrazol-1-carbothioamide (10a, 10b)

A mixture of the selected chalcones **3a**, **3c** (1 mmol) in absolute ethyl alcohol (20 ml) and thiosemicarbazide (0.09 g, 1 mmol) in glacial acetic acid (5 ml) was refluxed for 24 h. The reaction mixture was cooled and poured onto crushed ice; the formed solid was filtered off, washed and crystallized from ethyl alcohol to give the title compounds.

3.1.8.1. 4,5-Dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-5-(4-methoxyphenyl) pyrazol-1-carbothioamide (10a).

Mol. for.: $C_{20}H_{17}N_3O_4S$, M.wt. 395.43, yield 60%, mp 133–135 °C; Anal. Calcd: C, 60.75; H, 4.33; N, 10.63; S, 8.11. Found: C, 60.39; H, 4.15; N, 10.61; S, 8.09. IR (KBr, cm^{-1}): 3359 (OH, br s), 3171, 3046 (NH_2), 1698 (C=O, α-pyrone), 1618 (C=N), 1243 (C=S); ^1H NMR (DMSO-d_6, δ, ppm): 2.10 (2H, s, NH_2, D_2O exchangeable), 3.70 (3H, s, OCH_3), 3.74 (1H, dd, H-pyrazoline), 4.04 (1H, dd, H-pyrazoline), 5.32 (1H, dd, H-pyrazoline), 6.78–7.15 (8H, m, Ar-H), 14.30 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 38.55, 56.02, 66.72, 88.05, 114.16, 114.16, 117.57, 121.60, 125.56, 126.76, 128.02, 128.02, 128.43, 134.95, 150.21, 155.62, 158.71, 159.63, 161.16, 175.96; MS; *m*/*z* (R.A. %): (M^+) 395 (100%).

3.1.8.2. 4,5-Dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-5-(pyridin-3-yl) pyrazol-1-carbothioamide (10b).

Mol. for.: $C_{18}H_{14}N_4O_3S$, M.wt. 366.39, yield 82%, mp 215–217 °C; Anal. Calcd: C, 59.01; H, 3.85; N, 15.29; S, 8.75. Found: C, 58.98; H, 3.81; N, 15.18; S, 8.55. IR (KBr, cm^{-1}): 3412 (OH, br s), 3168, 3004 (NH_2), 2928 (–CH aliphatic stretching), 1703 (C=O, α-pyrone), 1609 (C=N), 1250 (C=S); ^1H NMR (DMSO-d_6, δ, ppm): 2.121 (2H, s, NH_2, D_2O exchangeable), 3.75 (1H, dd, H-pyrazoline), 4.14 (1H, dd, H-pyrazoline), 5.36 (1H, dd, H-pyrazoline), 6.68–8.85 (8H, m, Ar-H), 14.61 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 38.55, 66.74, 88.15, 117.53, 121.50, 123.43, 125.52, 126.82, 128.42, 133.71, 140.77, 147.04, 148.54, 150.23, 155.64, 159.53, 161.19, 175.96; MS; *m*/*z* (R.A. %): (M^+) 366 (6%), 365 (6%), 311 (100%), 301 (6%), 285 (87%), 284 (63%), 182 (32%).

3.1.9. Preparation of 5-Aryl-4,5-dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-*N*-phenyl-pyrazol-1-carbothioamide (11a–11c)

A mixture of **7a–7c** (2 mmol) and phenylisothiocyanate (0.13 g, 2 mmol) in dry acetone and few drops triethylamine was stirred at room temperature for 10–12 h. The formed precipitate was collected by filtration, washed with dry acetone and crystallized from methyl alcohol to give the desired compounds.

3.1.9.1. 4,5-Dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-5-(4-methoxyphenyl)-*N*-phenylpyrazol-1-carbothioamide (11a).

Mol. for.: $C_{26}H_{21}N_3O_4S$, M.wt. 471.53, yield 88%, mp 208–210 °C; Anal. Calcd: C, 66.23; H, 4.49; N, 8.91; S, 6.80. Found: C, 66.09; H, 4.41; N, 8.41; S, 6.56. IR (KBr, cm^{-1}): 3433 (OH, br s), 3300 (NH), 2926 (–CH aliphatic stretching), 1721 (C=O, α-pyrone), 1611 (C=N), 1249 (C=S). ^1H NMR (DMSO-d_6, δ, ppm): 3.62 (1H, dd, H-pyrazoline), 3.89 (3H, s, OCH_3), 4.33 (1H, dd, H-pyrazoline), 5.94 (1H, dd, H-pyrazoline), 6.64–7.45 (13H, m, Ar-H), 8.01 (1H, s, NH, D_2O exchangeable), 12.05 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 38.56, 55.93, 66.97, 88.01, 114.12, 114.12, 117.52, 121.53, 124.84, 125.54, 126.53, 126.53, 126.86, 128.01, 128.01, 128.45, 129.12, 129.12, 135.85, 137.17, 150.24, 155.65, 158.72, 159.51, 166.13, 179.6; MS; *m*/*z* (R.A. %): (M^+) 471 (0.1%), 336 (31%), 335 (38%), 229 (100%), 135 (94%), 121 (82%).

3.1.9.2. 4,5-Dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-5-(3,4,5-trimethoxyphenyl)-*N*-phenylpyrazol-1-carbothioamide (11b).

Mol. for.: $C_{28}H_{25}N_3O_6S$, M.wt. 531.58, yield 82%, mp 215–217 °C; Anal. Calcd: C, 63.26; H, 4.74; N, 7.90; S, 6.03. Found: C, 63.11; H, 4.71; N, 7.66; S, 6.13. IR (KBr, cm^{-1}): 3435 (OH, br s), 3290 (NH), 2936 (–CH aliphatic stretching), 1721 (C=O, α-pyrone), 1614 (C=N), 1235 (C=S). ^1H NMR (DMSO-d_6, δ, ppm): 3.42 (1H, dd, H-pyrazoline), 3.89 (9H, s, OCH3), 4.03 (1H, dd, H-pyrazoline), 5.44 (1H, dd, H-pyrazoline), 6.54–7.05 (11H, m, Ar-H), 7.76 (1H, s, NH, D_2O exchangeable), 13.35 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 38.51, 56.53, 56.53, 56.53, 67.57, 88.01, 114.23, 114.23, 117.52, 121.54, 124.72, 125.52, 126.53, 126.53, 126.76, 128.31, 129.12, 129.12, 137.17, 137.24, 137.84, 150.25, 150.62, 150.62, 155.64, 159.56, 166.12, 179.6; MS; *m*/*z* (R.A. %): (M^+−2) 529 (0.8%), 392 (4%), 226 (9%), 174 (5%), 106 (15%), 77 (28%), 44 (100%).

3.1.9.3. 4,5-Dihydro-3-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-*N*-phenyl-5-(pyridin-3-yl) pyrazol-1-carbothioamide (11c).

Mol. for.: $C_{24}H_{18}N_4O_3S$, M.wt. 442.49, yield 89%, mp 210–213 °C; Anal. Calcd: C, 65.14; H, 4.10; N, 12.66; S, 7.25. Found: C, 65.11; H, 3.99; N, 12.44; S, 7.15. IR (KBr, cm^{-1}): 3397 (OH, br s), 3061 (NH), 1703 (C=O, α-pyrone), 1610 (C=N), 1229 (C=S). ^1H NMR (DMSO-d_6, δ, ppm): 3.62 (1H, dd, H-pyrazoline), 4.23 (1H, dd, H-pyrazoline), 6.15 (1H, dd, H-pyrazoline), 7.23–8.44 (13H, m, Ar-H), 8.61 (1H, s, NH, D_2O exchangeable), 10.45 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (DMSO, δ, ppm): 38.56, 66.97, 88.01, 117.52, 117.52, 121.53, 123.84, 125.54, 126.53, 126.53, 126.86, 128.4, 128.4, 129.12, 129.12, 133.85, 137.17, 140.4, 148.5, 150.24, 155.65, 159.72, 166.13, 179.6; MS; *m*/*z* (R.A. %): (M^++1) 443 (0.18%), 228 (11%), 194 (18%), 135 (55%), 93 (90%), 77 (100%), 51 (56%).

3.2. Biological activity

3.2.1. Anticoagulant activity

3.2.1.1. In vitro anticoagulant activity. Seventeen 4-hydroxycoumarin derivatives were tested for their PIVKA-II production by HepG2 cells as indication for their anticoagulation activity. Coumarin anticoagulants antagonize VKOR, preventing vitamin K recycling and resulting in an accumulation of des-γ-carboxy prothrombin (DCP) or proteins induced by vitamin K antagonism (PIVKA-II). HepG2 (human hepatocellular carcinoma) cells are known to produce these proteins in response to warfarin treatment, which can be assayed using ELISA.

PIVKA-II production and hence anticoagulant activity is considered negative if the absorbance observed is below that produced by the 2 ng/ml standard.

HepG2 (human hepatocellular carcinoma) cells were purchased from ATCC and maintained in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C, 5% CO_2. The cells were seeded at 2.5×10^5 cells/well of 12 well plates and cultured 48 h prior to addition of treatments. The stock solutions and dilutions for warfarin and the tested compounds were freshly prepared on the day of the experiment. DMSO was used as the solvent for warfarin. Each experimental condition was tested in a single well. Wells were washed with Hank's balanced saline solution before the addition of 1.5 ml of reduced serum assay medium supplemented with warfarin or tested compounds. A no-treatment control was included in each assay. The solvents used were also tested at a final concentration of 0.2%.

At the end of the treatment period (24 h), 1 ml of supernatant was collected from each culture well and stored at −70 °C until tested using PIVKA-II ELISA (Diagnostica stago). The ELISA was performed according to the manufacturer's instructions. The samples were assayed neat and in duplicates.

3.2.1.2. In vivo anticoagulant activity. The experiment was carried out on 60 albino male mice weighing from 20 to 25 g. The mice were divided into 10 equal groups of six mice each. Mice within group (1) were kept as control and received propylene glycol in distilled water only, whereas those of groups (2) to (9) were given the tested compounds **4a**, **5a**, **6a**, **7c**, **8b**, **9b**, **10b** and **11c** and mice in the last group (10) were given warfarin as the standard control. The tested compounds and warfarin suspended in propylene glycol were administered orally with daily dose 150 mg/kg for three successive days. Blood samples were taken after 24 h from the last injection and coagulation time (CT)[29] and prothrombin time (PT)[30] were determined.

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