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Some Experimental Studies on the Anticoagulant Activity of the Synthetic Coumarin Derivatives

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Abstract

Intensive research efforts have been devoted to the design and synthesis of 4-hydroxycoumarin derivatives as anticoagulants. The main purpose of this study was to report the synthesis of some coumarin-type derivatives, including, 4-aryl-1,2-dihydro-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxopyridin-3-carbonitrile (3, 4). The structure of the synthesized compounds has been verified on the basis of literature data and spectroscopic measurements such as NMR, MS and IR. In addition, there were two objectives for the study: first, to test these synthetic derivatives *in vivo* for their anticoagulant effects in the laboratory male mice (*Mus musculus swiss albino*). Second, to compare between the *in vivo* activity of these synthetic derivatives and that of Warfarin (CAS 81-81-2), which is the most commonly used anticoagulant. Prothrombin time (PT) was used as the value to compare the anticoagulant properties of the synthesized compounds (1, 3, 4) and warfarin. The time for plasma to solidify following the addition of warmed thrombokinase suspension was taken as the PT. Results of this study revealed that the most potent compound of the synthesized derivatives was 4-(3-bromo-phenyl)-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxo-1,2-dihydro-pyridine-3-carbonitrile (4), which shows higher anticoagulant activity ($PT_{(s)}$ 21.30) than warfarin ($PT_{(s)}$ 14.60). However, Anticoagulant activity was also associated with toxicity by the all synthesized compounds. Conclusion and practical importance: Synthesized coumarins (3 and 4) increased PT when compared to saline treated control group and other coumarins. They are potentially antithrombotic drug candidates for further elaboration. Compounds 3 and 4 need to be further tested for the side effects so that they can be introduced into clinical trials.

Keywords: Coumarine derivatives, Hyroxycoumarin, Warfarin, Anticoagulant activity

1. Introduction

Coumarins are one of the most significant families of natural product compounds and can be found in many plants as secondary metabolites. Chemically, coumarins belong to the subgroup of lactones (Nikhil *et al*., 2012). There is a large number of coumarin derivatives, namely natural and synthetic coumarin, which associated with various types of biological activities, such as anti-inflammatory, anticancer, antioxidant, anti-HIV, as well as anti-coagulant (Murray *et al*., 1982; Kostova, 2005; Kontogiorgis and Hadjipavlou-Litina, 2005; Yuce *et al*., 2009; Fernanda *et al*., 2015). Coumarins and their derivatives are principal oral anticoagulants, and this is attributed to their competitive inhibition effect on vitamin K in the biosynthesis of prothrombin (Beillerot*et al*., 2008; Ozkan*et al*., 2010). Clinically, coumarine derivatives are the precursors of several anticoagulants, particularly warfarin, which is the most commonly used oral anticoagulant medication. All of the above information has been the motivation to conduct the current study. Chemically, coumarins are extremely variable in structure due to the different types of substitutions in their basic structure. In this context, the aims of this study were to report the synthesis of some coumarin derivatives, including, 4-aryl-1,2-dihydro-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxopyridine-3-carbonitriles and then to evaluate their anticoagulant activities, in comparison to that of commercially anticoagulant drug warfarin.

2. Metarial and Method

All reactions were performed under an atmosphere of argon in oven-dried glassware. Anhydrous solvents for reactions were obtained by filtration through activated alumina or by storage over molecular sieves (4 Å). Phenol (Sigm-Aldrich, Switzerland), 4-hydroxy-coumarin (Sigm-Aldrich, Switzerland), oxychloride-phosphorus (Sigm-Aldrich, Switzerland), chloride-zinc anhydride (Sigm-Aldrich, Switzerland), malonic acid (Sigm-Aldrich, Switzerland), glacial acetic acid (Sigm-Aldrich, Switzerland), sodium carbonate (Sigm-Aldrich, Switzerland), 3-nitrobenzaldehyde (Sigm-Aldrich, Switzerland), 3-bromobenzaldehyde (Sigm-Aldrich, Switzerland), ethyl cyanoacetate (Sigm-Aldrich,

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Switzerland), ammonium acetate (Sigm-Aldrich, Switzerland), ethanol (Sigm-Aldrich, Switzerland), methanol (Sigm-Aldrich, Switzerland) and warfarin (Merck & Co, Germany) were used as reagents in the chemical reactions. IR spectra were recorded with the IR spectrophotometer (IR Perkin Elmer 297). The naming of the structures was done by the ChemDraw program, 545.^{1}H-NMR spectra were recorded on a 300 MHz NMR spectrometer instrument (Bruker AC 300). Mass spectra were recorded on an AutoSpeq Q VG with ionization energy of 70ev.

*2.1. Synthesis of 4-hydroxycoumarin (**1**)*

Firstly, phenol (0.01 mol), malonic acid (0.01 mol), oxy-chloride phosphorus (40 ml) and zinc chloride-anhydride (30 g) were heated in a water bath at 70 °C for 12 hours. Thereafter, this reacted mass was cooled and poured into ice. Then, the solid cooled mass was dissolved in 10% sodium carbonate and filtered. 4-Hydroxycoumarine, white precipitate, was obtained by acidification of the filtrate. The reaction yield was 98%. The melting point of the synthesized compound corresponds to the literature data 211 – 213 ^{0}C (Naveen *et al*., 2006).

*2.2. Synthesis of 3-acetyl-4-hydroxy-coumarin (**2**)*

12.5 ml of $POCl_3$ was added to the 8.1 ml solution of 4-hydroxy-coumarin in 45 ml of glacial acetic acid. The mixture was refluxed for 2 hours. After cooling, a voluminous white precipitate was obtained which was filtered and dried. The precipitate was recrystallized from methanol and 3-acetyl-4-hydroxycoumarine was obtained as a white to yellow substance with needle-shaped crystals, as shown in Figure 1. The melting point corresponds to the data in the literature 210 ^{0}C (Al-ayed, 2011; Dholakia *et al*., 1968). Yields 80%.

Figure 1. Crystals of compound **2**.

*2.3. Synthesis of 6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-4-(3-nitro-phenyl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**3**)*

3-Acetyl-4-hydroxy-coumarin (1g, 5mmol) was mixed with 0.75g (5 mmol) 3-nitro-benzaldehyde in 20 ml ethanol. In this mixture, 0.57ml ethyl acetate and 0.75g (10mmol) ammonium acetate were added. The mixture was refluxed for 4hours. The yellow precipitate (Figure 2) was recrystallized from ethanol or methanol. Melting point 223 ^{0}C. Yields 72%. IR (KBr, ʊ cm^{-1}): 3450. 3300. 3030, 2950, 2200, 2000, 1750, 1520, 1340.^{1}H-NMR (300 MHz, DMSO): δ 5.63 (s, 1H, C-H), 7.20-8.23 (m, 9H), 13.33 (s, 1H, OH). HRMS (ESI): m/z [M + H]$^{+}$ calcd for $C_{21}H_{12}N_3O_6$ 402.0681, found 402.0683.

Figure 2. Crystals of compound **3**.

*2.4. Synthesis of 4-(3-bromo-phenyl)-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**4**)*

3-Acetyl-4-hydroxy-coumarin (1g, 5 mmol) was mixed with 3-bromo-benzaldehyde (0.95g, 5 mmol) in 20 ml ethanol, and another 0.57 ml ethyl acetate and 0.75g (10 mmol) ammonium acetate was added in the mixture. The mixture was refluxed for 5 hours. White precipitates were recrystallized from ethanol to obtain white crystals as in Figure 3. Melting point 215 ^{0}C. Yields 64%. IR (KBr, ʊ cm^{-1}): 3450, 3030, 2950, 2240, 1750, 750-800.^{1}H-NMR (300 MHz, DMSO): δ 5.71 (s, 1H, C-H), 7.19-8.20 (m, 9H), 13.50 (s, 1H, OH). HRMS (ESI): m/z [M + H]$^{+}$ calcd for $C_{21}H_{12}BrN_2O_4$434.9903, found 434.9969.

Figure 3. Crystals of compound **4**.

2.5. Animals and treatment protocol

Male laboratory mice of the type *Mus musculus swiss albino* aged 12-17 weeks (Figure 4A) were used to measure the anticoagulant effect of the synthesized compounds. Male laboratory rats weighing 130–160 g were obtained from the Experimental Animals Unit of the Departament of Biology at the University of Prishtina. The study was approved by the Prishtina University Committee. The animals were housed in stainless steel cages in a room at 23 ± 2 ^{0}C under 12 h dark/light cycles. After arrival, all the rats were fed standard rat chow for 7 days. Rats were given free access to food and water.

The synthesized compounds were mixed with 0.9% NaCl solution. Gastric tube was used for oral adminestration of prepared -solutions of warfarinë (1 mg/kg) and synthesized compounds **3** and **4** (20 mg/kg), respectivly (Figure 4B). The rats were then allowed to stand for 24 h in order for the substances to achieve the desired effect before analysis.

A

B

Figure 4. **A**) Mice (*Mus musculus Swiss albino*), **B**) Treatment with intragastric tube.

Then the rats were randomly divided into 5 groups as; Group I: 4-hydroxy coumarin (**1**) (n = 5), Group II: 6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-4-(3-nitro-phenyl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**3**) (n = 5), Group III: 4-(3-bromo-phenyl)-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**4**) (n = 5), Group IV: warfarin (n = 5), Group V: control (n = 5).

2.6. Drug administration and prothrombin time

Drugs dissolved in saline were administered to the coumarin treated groups (Groups I, II and III) and warfarin treated group (Group IV) by oral gavage route for a single time with a dose of 20 mg/kg and 1 mg/kg, respectively (Figure 4B). For the control group (Group V), the vehicle used was saline solution. After 24 h, the animals were anaesthetized with ethyl ether by inhalation and blood samples were immediately collected through cardiac puncture into a tube containing 1:9 volume 3.8% g sodium citrate. The blood was then centrifuged (Denley DJB Labcare Ltd., BS 400, Buckinghamshire, UK) for 15 min. The separated plasma was transferred to a test tube kept in a water bath (Clifton Nickel-Electro Ltd., Digital Shaker Bath, North Somerset, UK) at 37 ^{0}C. The anticoagulant activity of coumarins was determined by using Stago-Neoplastine CI Plus Kit (Diagnostica Stago, Asnieres, France). The time for plasma to solidify following the addition of warmed thrombokinase suspension was taken as the prothrombin time (PT), as described by Ozkan,*et al*. (2010).

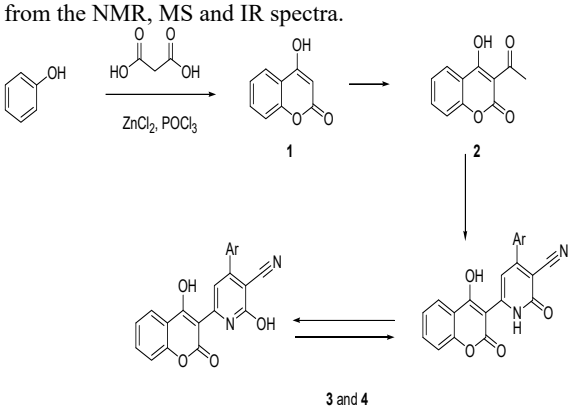
2.7. Statistics Analysis

All statistical analysis was carried out using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA. USA). Groups of data were compared with two-tailed t test. Values of $p < 0.05$ or $p < 0.01$ were regarded as significant or highly significant, respectively.

3. Results and Discussion

3.1. Synthesis of coumarine derivatives

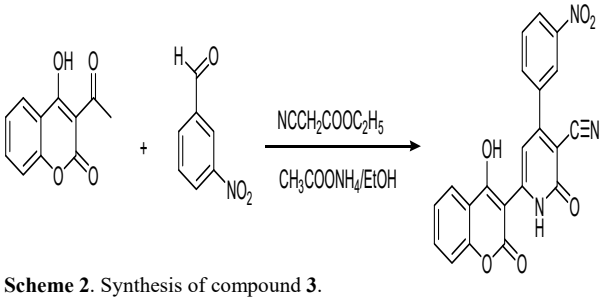
4-Hydroxycumarine derivatives are synthesized according to scheme 1, using procedure described by Abdelhafez, *et al*., (2010). Structure of the synthesized compounds has been verified on the basis of data obtained from the NMR, MS and IR spectra.



Scheme 1. The strategy for the synthesis of coumarin derivatives with anticoagulant properties.

By reaction of phenol with malonic acid in the presence of oxychloride-phosphorus and anhydride zinc chloride, compound **1** was obtained. The structure of the synthesized compound **1** is verified by the melting point. The melting point corresponds to the literary data 211-213 ^{0}C (Naveen *et al*., 2006). By reaction of 4-hydroxycoumarin with glacial acetic acid in the presence of $POCl_3$, a white to yellow precipitate of **2** was obtained according to the above reaction (Scheme 1). Also, the melting point of **2** corresponds to the literary data 210 ^{0}C (Al-ayed, 2011; Dholakia *et al*., 1968). In the IR spectrum of this compound, a characteristic signal in the interval 3100-2500cm^{-1} has appeared which has resulted from the stretching vibrations of the group ט (-OH). The stretching vibrations ט (C-H) of the aromatic system and the ט (C-H) stretching vibrations of the methylene group are displayed with weak intensities at 2750 cm^{-1} and 2550 cm^{-1}. In the range 1800-2000cm^{-1}, a series of weak absorptions have appeared resulting from overtones that are characteristic of the aromatic ring. Also, the peak in the range 1600cm^{-1} is characteristic of the stretching vibrations ט (C=C). At 1750 cm^{-1}, a characteristic adsorption of the (C=O) group of carbonyl compounds has been shown, whereas the peak in the range 1250 cm^{-1} is characteristic of the stretching vibrations of the C-O bond.

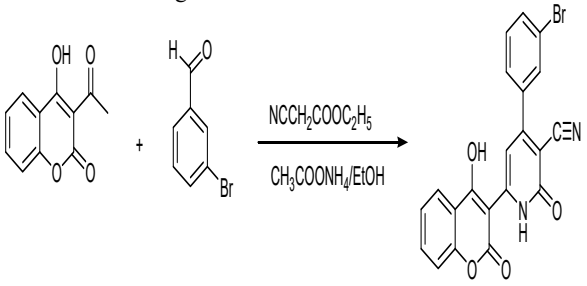
The reaction of the compound **2** with 3-nitro-benzaldehyde in the presence of ethylcyanoacetate, a compound **3** as yellow precipitate was obtained according to the reaction shown in Scheme 2.



Scheme 2. Synthesis of compound **3**.

In the IR spectrum of this compound these characteristic absorptions have appeared. At 3450 cm^{-1} the absorption peak for vibration of N-H group is shown, while at 3300 cm^{-1} the absorption peak of the OH group is shown. Aromatic ט (C-H) stretching vibrations are displayed at 3030 cm^{-1} and aliphatic stretching (CH) vibrations at 2950 cm^{-1}. At 2200 cm^{-1} a weak peak has appeared as a result of (C≡N) stretching vibrations. At 1750-2000 cm^{-1} the characteristic overtones for the aromatic system have appeared. Vibrations ט (NO_2) asymmetric stretching and ט (NO_2) symmetrical stretching are shown at 1520 cm^{-1} and 1340 cm^{-1}.

By reaction of **2** with 3-bromo benzaldehyde in the presence of ethylcyanoacetate, ammonium acetate and ethanol a new product **4** with crystal structure was obtained according to the reaction shown in Scheme 3.



Scheme 3. Synthesis of compound **4.**

In the IR spectrum of this compound, these characteristic absorptions have appeared. At 3450 cm^{-1} a peak with average intensity characteristic for the ט (N-H) stretching vibrations of the secondary amines is shown. At 3030 cm^{-1} ט (C-H) aromatic stretching vibrations appeared and at 2950 cm^{-1} (C-H) aliphatic vibrations. At 2240cm^{-1} an intense peak has appeared resulting from (C≡N) stretching vibrations. At 1725 cm^{-1} a characteristic peak for stretching vibration (C=O) has appeared, whereas two symmetrical signals in the range 750 -800 cm^{-1} are characteristic of monosubstituted aromatic ring.

3.2. Prothrombin time

PT values for the groups are given in Table 1. PT was higher in all groups when compared to control; however, it was significantly higher in the **4** treated and warfarin treated groups than in the control group ($p < 0.01$).

Thromboembolism is an important cause of morbidity and mortality. To prevent vascular death ensuing from thrombosis, oral administration of anticoagulant drugs is one of the most important treatments. Vitamin K antagonists such as warfarin, acenocoumarol and phenprocoumarin are the main anticoagulant drugs producing an anticoagulant effect by interfering with the cyclic inter-conversion of vitamin K and its 2,3-epoxide (D'Andrea*et al*., 2008). Warfarin is the most widely used coumarin oral anticoagulant because its onset and duration of action are predictable. The maintenance dosage of warfarin varies between 1 and 30 mg/kg daily (Hirsch *et al*., 1992). After 24–48h of starting warfarin, the INR (International Normalized Ratio) begins to rise (Gage and Milligan, 2005). Coumarins exert their effects *in vivo* only after a latent period of 4–12 h, and their effect lasts for 1.5–5 days (Hirsch and Fuster, 1995). The anticoagulant activity of coumarins blocking the prothrombin biosynthesis by inhibition of vitamin K-epoxide reductase is closely dependent on the presence of a hydroxyl group in position 4 and a highly lipophilic substituent in position 3 of the benzopyrano moiety (Manolov and Danchev, 1995). On the other hand, despite the lack of the aforementioned structural requirements, a similar mechanism of action is reported to take place for the anticoagulant 1,3-indandione derivatives (Bruno *et al*., 2006; Arora and Mathur, 1963).

From the preliminary anti-thrombotic activity screening studies done in our laboratory, we decided to use a single dosage of 20 mg/kg and blood samples were collected 24h after the administration of the synthesized coumarins. As shown in Table 1, the PT of all groups increased. However, the PT was significantly higher in the **4** treated group when compared to the warfarin group.

Table 1: Effect of the coumarins derivatives on prothrombin time of the rats 24 h after single oral administration.

	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
Prothrombin time (PT) (s)	11.30	20.80*	21.30*	14.60*	9.16
Standard deviation/ Standard error	0.63/0.30	0.54/0.24	0.38/0.15	0.39/0.16	0.15/0.07

Values are given as mean and standard deviation (SD), standard error (SE). Group I was treated with 4-hydroxycoumarin (20 mg/kg), Group II with 6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-4-(3-nitro-phenyl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**3**) (20 mg/kg),4-(3-bromo-phenyl)-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**4**) (20 mg/kg), Group IV with warfarin (1 mg/kg), while Group V was treated with saline (control).* Significant difference between control and treated sample: $p < 0.01$ (by 2-tailed t-test); $n = 5$.

Compound **4** is a potential compound as an antithrombotic drug for further elaboration.

After injection of synthesized compound **3** in mice, we have seen stereotypical behavior (increased locomotors activity repeated in uninterrupted series) as shown in Figure 5A. Also, in animals, treated with compound **3**, changes in the intestinal tract were observed, which appeared with diarrhea (liquid feces) (Figure 5B). The increased secretion of the eye and edema of the eyelids was observed as another symptom. Mice treated with compound **3** had milky white secretion from the tear duct and increased eyelid edema up to total eye closure (Figure 5C).

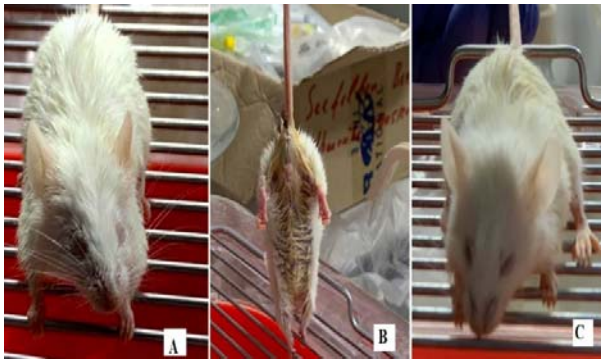


Figure 5. Some of the toxic effects presented in the tested mice, **A**) edema of the eyelids, **B**) diarrhea, **C**) increased secretion in the eye.

Mice treated with compound **4** had almost identical intestinal symptoms. All other behavioral or visual system-related symptoms were not present in mice treated with compound **4**.

The commercial drug, warfarin, had the same symptomatology as that of the group of mice treated with compound **3**, except that the symptoms were not as high. In particular, no changes in behavior were recorded.

Based on these results as well as the results obtained from Table 1, we see that these derivatives show *in vivo* anticoagulant potential. On the other hand, in addition to anticoagulant activity, these derivatives have been associated with high toxic effects, which is another problem that requires further study.

4. Conclusion

In conclusion, the PT value of 6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-4-(3-nitro-phenyl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**3**) and 4-(3-bromo-phenyl)-6-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2-oxo-1,2-dihydropyridin-3-carbonitrile (**4**) were higher than that of warfarin. Based on the anticoagulant activity, they are a potentially antithrombotic drug candidate for further elaboration. As a result of this work, we concluded that these derivatives showed satisfactory anticoagulant potential, but that anticoagulant ability was closely related with toxicity. We recommend working on these derivatives by changing their structures with the aim to decrease their toxic effects.

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