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Original Research Article

Synthesis of 4-hydroxycoumarin and 3-acetyl-4-hydroxycoumarin in the presence and absence of ultrasonic bath and evaluation of their anticoagulant effects

Sanaz Firoozi, Sabah Salahvarzi*, Zeinab Dadgar

Department of Chemistry, Faculty of Science, Khorramabad Branch, Islamic Azad University, Khorramabad, Iran

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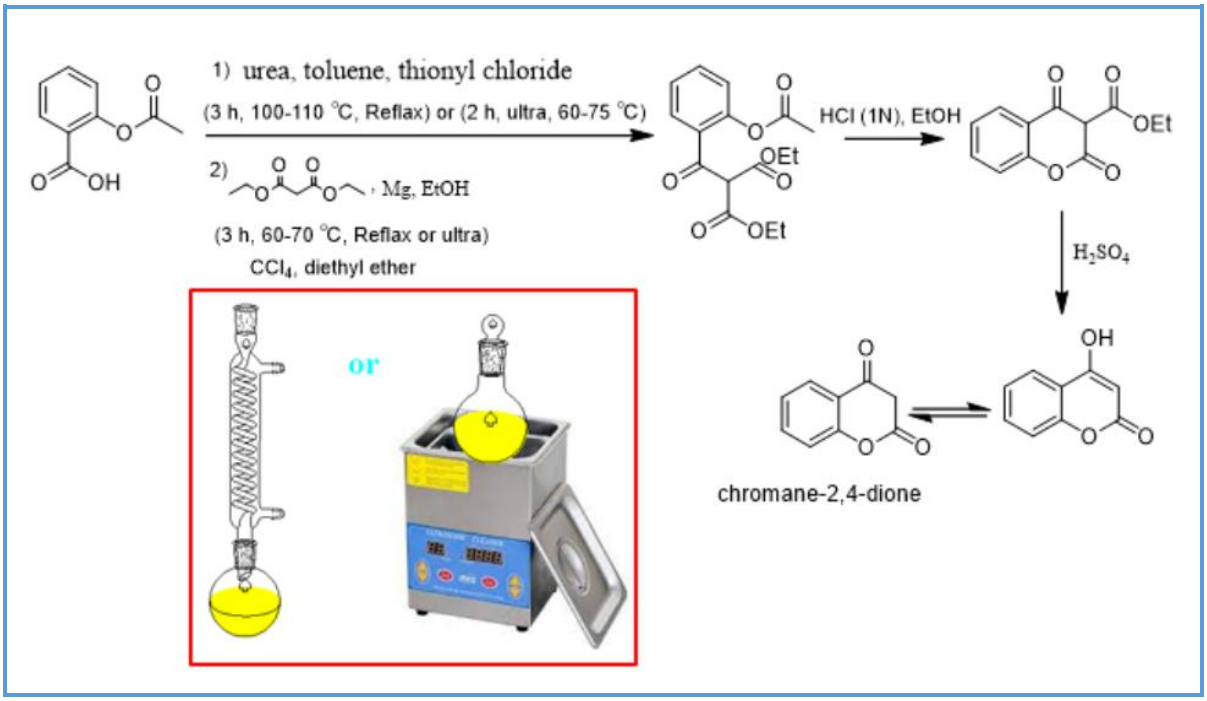
ABSTRACT

Biochar is a cost-effective and porous material with high carbon content. It is considered as an effective supporting matrix owing to its high specific surface area and notable ion exchange ability. In this work, a porous biochar support was fabricated from pistachio residues using pyrolysis procedure. Subsequently, various crystalline phases and morphologies of MnO_2 were deposited onto the biochar support through chemical protocols with $Mn(Ac)_2$, $KMnO_4$, and $MnSO_4$ as Mn source. The N_2 adsorption-desorption experiments were employed to characterize the porosities and specific surface areas of the synthesized nanocomposites. It is found that the γ-MnO_2/biochar composite possessed the higher surface area than the δ-MnO_2 and α-MnO_2 samples. The adsorption features of the composite materials in the removement of target dye from aqueous solution were also examined. Based on the experimental results, the γ-MnO_2/biochar sample showed the highest efficiency for removal of target dye. In addition, the experimental data exhibited a good correlation (R^2 greater than 0.99) with the pseudo-second-order kinetic model, indicating a chemical adsorption approach for dye adsorption.

Corresponding author, email: sabahsalahvarzi@yahoo.com (S. Salahvarzi).

Graphical Abstract



Introduction

Anticoagulants are compounds that prevent blood from clotting. Blood clotting is a process that causes blood to clot. This process converts fibrinogen to fibrin through internal and external pathways. It also activates coagulation factors and causes platelet aggregation. Anticoagulants are naturally found in the bodies of leeches and insects that feed on the blood of other organisms, such as mosquitoes.

Coumarin derivatives are a class of anticoagulants that act as vitamin K antagonists. Coumarins are an old group of a family of natural compounds. Coumarin was initially isolated from tonka beans in 1822. Coumarin and its derivatives were isolated from sweet clover, bison grass, and an insect called Woodruff. Coumarins are benzo (alpha) pyrenes formed through shikimic acid. With the exception of a few rare non-substituted coumarins, all hydroxy and methoxy coumarins have been replaced in the V position [1-4]. Coumarins are involved in plant growth hormones and growth regulators. They also play a role in photosynthesis, respiratory control, and defense against infection. Coumarins have gained a lot of attention because of their medicinal properties. Coumarin and its derivatives have long been used in biology, medicine, and polymer science [5-11]. Currently, coumarins are used in the perfume industry, cosmetics, cigarettes, alcoholic beverages, and laser pigments. Coumarins are widely consumed in the human diet, for example carrots, celery, etc. They are fluorescent compounds and this property is widely used in a number of biochemical techniques. Simpler coumarins are also used as pigments in sunscreens [12,13]. Coumarins and their derivatives are generally known as a heterocyclic group which are very attractive due to their wide range of biological applications such as anticoagulant, anti-inflammatory, enzymatic inhibitory properties,

antioxidant activity, anti-HIV, anti-cancer, epilepsy treatment, anticonvulsant as well as their role as anti-allergy, antimicrobial and photodynamic activity [14-18]. Coumarin itself has no anticoagulant activity because in position 4, there is no hydroxy group attached to carbon 4. The pharmacological activity of coumarins depends on the side chain and replacement around their central ring structure. Thus, unlike coumarin derivatives, coumarin has no anticoagulant effect. However, in low doses (typically 7 to 10 mg daily) coumarin is used as a stenoty for vein and blood flow health.

As mentioned above, coumarin derivatives have numerous applications in medical sciences, biomedical research, and many industries. Therefore, many efforts are being made to develop new and more practical methods for the synthesis of these compounds.

Numerous studies describe several methods for the synthesis of coumarin derivatives, including Perkin density, Pechmann, Knoevenagel density, Wittig reaction, Baylis-Hillman reaction, Michael addition, Kostanecki reaction, vinyl phosphonium salt-mediated electrophilic reaction, and Heck-lactonization reaction, etc. The most widely used method for their synthesis is the Pechmann reaction, which involves compression between phenols and beta-keto esters in the presence of an acid catalyst [19-21]. Recently, in many studies, nanoparticles have been used as catalysts for the synthesis of coumarin and chromone derivatives [22,23]. A series of oxocyclopenta[c]chromenes were synthesized via three component reaction of 4-hydroxycumarin, dialkyl acetylenedicarboxylate, α-bromo ketones, and triphenylphosphine in the presence of catalytic amount of Fe3O4 magnetic nanoparticles (MNPs) (15 mol%) under solvent-free conditions at 70 °C [22]. Coagulation indicators measure the amount and function of certain proteins in the blood. These proteins are called coagulation or clotting factors. They are an important part of blood clot formation. Prothrombin, thrombin and thromboplastin are considered as important indicators of blood coagulation.

In the present study, Knoevenagel method is used for the synthesis of 4-Hydroxycoumarin and 3-acetyl-4-Hydroxycoumarin, which use two different synthetic methods. Likewise, each method is performed in two conditions of reflux and ultrasound bath and the methods are compared with each other and the advantages and disadvantages of each of them are examined. Coagulation indices are measured in the presence of normal saline (in the control group) and different concentrations of 4-Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin in the laboratory.

Experimental

Materials and equipment

All materials and solvents used in this study were provided by the German company Merck. The nuclear magnetic resonance device (NMR, BRUKER 400MHZ model) was used to determine the structure of the molecules. RX I Perkin ELMER infrared Fourier transform (FT-IR) was used to identify the functional groups of structures. To evaluate the products' purity with the help of TLC and place them in the UV cabinet device, Azar Ashna Nab model was used in the range of 365-254 nm. The SONICA model ultrasonic bath was used to disperse and smooth the particles in the solution and increase the quality of the solution. The Universal Pars Azma centrifuge was used to separate solid particles from the liquid. To weigh solids, Sartorius digital scale with accuracy of 0.001 was used.

Synthesis of aspirin

To prepare and synthesize coumarin derivatives, aspirin is a required raw material. The following method was used to synthesize aspirin. In a 100 ml Erlenmeyer flask, we combined 40 g (289.6 mmol) of salicylic acid and 30 ml (317.37 mmol) of acetic anhydride with 3 ml of sulfuric acid. We then put the flask in a water bath at 60 °C for 15 minutes. After that, the reaction vessel was placed in an ice bath until white crystals appeared. We purified aspirin with recrystallization using ethanol and water. We formed pure white aspirin crystals, and then we smoothed and dried the precipitate at ambient temperature. The product had a melting point of 134 °C.

Synthesis of 4-hydroxycoumarin and 3-acetyl-4-hydroxycoumarin

For the preparation and synthesis of coumarin derivatives, two different methods were used and both methods were performed under different conditions, once under reflux and again by ultrasound bath.

Synthesis of 4- hydroxycoumarin under reflux / ultrasonic bath conditions (method 1)

In the first flask, we first combined aspirin (6 g, 33.3 mmol), urea (0.04 g) and toluene without water (5 ml) while stirring in an ice bath at a temperature between 10 and 15 °C and shook well. In the next step, thionyl chloride (4.76 ml) was added dropwise to the solution while stirring. The flask was then placed under reflux for 2-3 hours with a stirrer (in an oil bath at 100 to 110 °C, 3 h) or an ultrasonic bath (temperature between 60-75 °C, 2 h). In another flask, diethyl malonate (5.34 ml and 35.31 mmol), ethanol (6.48 ml), magnesium (0.84 g), carbon tetrachloride (0.32 ml), and diethyl ether (40 ml) were combined well by magnetic stirrer. The mixture was then placed in an oil bath at 60 to 70 °C for 3 hours under reflux or ultrasonic bath. After that, the two flasks were combined and placed on a stirrer for half an hour to get mixed, and then hydrochloric acid solution (one normal, 24 ml) and three drops of concentrated sulfuric acid (as a catalyst) were added to the solution. A pale-yellow precipitate formed with needle-shaped crystals. After smoothing the precipitate, it was placed in an oven at 80 °C to dry.

The efficiency of precipitates formed in reflux and ultrasonic bath methods was 57.96% and 90.07%, respectively. Also, the melting point of the product was obtained between 211 °C and 213 °C in both methods.

Synthesis of 3-acetyl-4-hydroxycoumarin and its conversion to 4-hydroxycoumarin under reflux / ultrasonic bath conditions (method 2)

In this section, two 50 ml flasks were selected, one for ultrasound bath and the other for reflux. In each flask, combined aspirin (18 g, 100 mmol) with urea (0.12 g) and toluene without water (14 ml) while stirring in an ice bath at a temperature between 10 °C and 15 °C and we shook them. In the next step, thionyl chloride (14.3 ml) was added dropwise to the solution during stirring, and then the flask was placed under reflux for 2-3 hours (in an oil bath at 100 to 110 °C for 3 hours) or placed under ultrasonic bath (with a temperature between 60 °C and 75 °C for 2 hours). After the reaction, the flask was cooled to room temperature, and then the product was placed in the oven at 72 °C to dry. The formed precipitate was brick red. Following the experiment, ethyl acetate (13 ml, 102.36 mmol) was poured into a 50 ml three-neck flask, and then one of the necks was blocked by the condenser and the other by a cork. The

third neck was connected to the decanter using a cork. The end of the condenser and the opening of the decanter were both connected to a nitrogen-filled balloon. We kept the whole system closed with Teflon tape to minimize the leakage of nitrogen out. The reaction vessel was cooled to 5 °C using an ice bath. Sodium solution (40%, 13.4 ml) was then added to the flask with a stirrer and the temperature was kept between 5 and 15 °C. Afterwards, the brick red precipitate of the first stage was added to the reaction vessel. The reaction mixture was stirred for one hour at 0-5 °C. After cooling the reaction vessel, sodium hydroxide solution (40%, 11 ml) was added and stirred at 35 °C for an hour, and then 40 ml of distilled water was added and the aqueous layer was washed with saturated sodium chloride solution. The resulting precipitate was smoothed and washed with water. The precipitate was dried at 80 °C in the oven. The formed precipitate was light yellow 3-Acetyl-4-Hydroxycoumarin. The efficiency of precipitate in reflux and ultrasonic bath methods was 94.57% and 81.89%, respectively.

To convert 3-Acetyl-4-Hydroxycoumarin to 4-Hydroxycoumarin in the next step, 3-acetyl-4-hydroxy coumarin (0.8 g, 3.9 mmol), concentrated sulfuric acid (24 ml), distilled water (5 ml) and ethanol (12 ml) were combined in a flask and the mixture was placed under reflux/ ultrasonic bath for an hour. The mixture vessel was then cooled to 10 °C. The solution was filtered, and then the precipitate was washed with distilled water to remove excess sulfuric acid. The formed precipitate dried at ambient temperature and a yellow crystalline precipitate was formed. After recrystallization with ethanol, a white crystalline precipitate was obtained. The precipitate efficiency in reflux and ultrasonic bath methods was 81% and 78.1%, respectively.

Clinical method

To perform coagulation test, different concentrations of test samples must be prepared. 4-Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin powders were dissolved in 0.9% normal saline at 80 °C and 40 °C, respectively, to make concentrations of 2%, 5%, and 10% to be used for *in vitro* studies. The present study was performed experimentally and *in vitro*. In fact, the tested samples were a total of 25 blood samples taken from 5 healthy males in the age range of 37-25 years, which were divided into control (5 samples) and experimental (5 samples per concentration) groups. Coagulation indices including prothrombin time, relative thromboplastin time, coagulation time in the presence of normal saline (in the control group) and different concentrations of 4-Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin were measured *in vitro*. All experiments in samples containing extract and carrier were performed in duplicate.

The data obtained from the study of coagulation indices was analyzed as the mean ± standard error of the mean (SEM) for blood samples from 3 individuals in each group. One-way analysis of variance (ANOVA way-One) followed by Tukey post hoc test was used for statistical analysis in multiple comparisons. In all analyzes, p values less than 2.5 were considered significant.

Results and discussion

FT-IR analysis, ^{13}C-NMR, and ^{1}H-NMR were used to identify and confirm the final structure of the products and the results of the spectra are given in the attached file.

Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance spectroscopy (^{13}C-NMR, ^{1}H-NMR)

The results of IR and NMR spectroscopy and the efficiencies of all products are presented in Table 1. Furthermore, the main spectra are available in the attached file.

Aspirin

Figure 1 displays the reaction of salicylic acid with acetic anhydride. IR spectroscopy is used to identify the final structure of the reaction product. In IR spectroscopy, the peak observed at 2994.24 cm^{-1} refers to the tensile vibrations of OH carboxylic acid. Tensile vibrations of aliphatic and aromatic C-H bonds are in the range of 2800-2870.06 cm^{-1}. The peak generated in the range of 1686.50 and 1752.16 cm^{-1} refers to the tensile vibrations of acidic and ester carbonyl, respectively. The carbon-carbon tensile vibrations of the aromatic ring have been appeared in the range of 1456.84-1605.26 cm^{-1}.

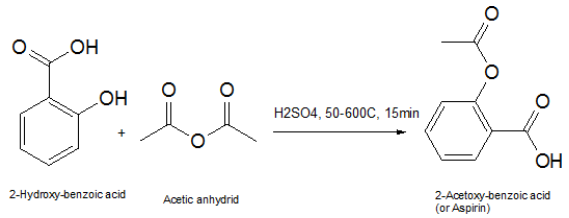


Figure 1. Reaction of salicylic acid with acetic anhydride for preparing aspirin

Hydroxycoumarin under reflux / ultrasonic bath conditions (method 1)

Figure 2 depicts the reaction of coumarin and diethyl malonate with a molar ratio of 1:1 under reflux and ultrasonic bath conditions. IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy were used to identify the final product structure of each reaction.

In IR spectroscopy, the peak 3232.49 cm^{-1} indicates the tensile vibrations of phenolic OH. Tensile vibrations of C=O were observed in 16778.41 cm^{-1}. Tensile vibrations of aromatic C=C appeared in 1453.22-1605.59 cm^{-1}. Dimethyl sulfoxide (DMSO) was used in ^{13}CNMR spectroscopy, which showed a seven-point peak of solvent carbon in the range of 39.3-40.6 ppm. The peaks observed at 160.63 and 172.4 ppm refer to C_2 and C_4 carbon, respectively. The peak corresponding to C_3 was shown at 113.3 ppm. The peaks observed at ppm 136.7-117.9 refer to aromatic ring carbon (C5-C8). Dimethyl sulfoxide was used in ^{1}H-NMR spectroscopy, and the peak for solvent hydrogens was 2.518 ppm. In the range of 10.30 ppm, the peak was related to the hydrogen of the OH group. Aromatic ring hydrogens appeared in the range of 6.93-7.522 ppm. The unique peak of 3.27 ppm referred to carbon 3 hydrogens. There were also a number of weak peaks that had been removed even after purifying the sample and were present in the sample direction which were probably related to the hydrolyzed open form of the product (Figure 3).

FT-IR, ^{13}C- and ^{1}H-NMR methods were used to identify and confirm the structure of the product created by ultrasonic bath method. In IR spectroscopy, a peak of 3235.80 cm^{-1} indicated the tensile vibrations of phenolic OH. The peak 2862.74-3064.42 cm^{-1} showed the presence of tensile vibrations of aromatic CH and CH of heterocyclic ring. C=O tensile vibrations were observed at the peak 1683.91 cm^{-1} for the molecule. The tensile vibrations of the C=C aromatic ring appeared in 1483.37-1613.19. Dimethyl sulfoxide was used in ^{1}HNMR spectroscopy, and the peak for solvent hydrogens was 2.53 ppm. Aromatic ring hydrogens appeared in the range of 6.92-7.82 ppm. The unique peak of 3.27 ppm referred to carbon 3 hydrogens.

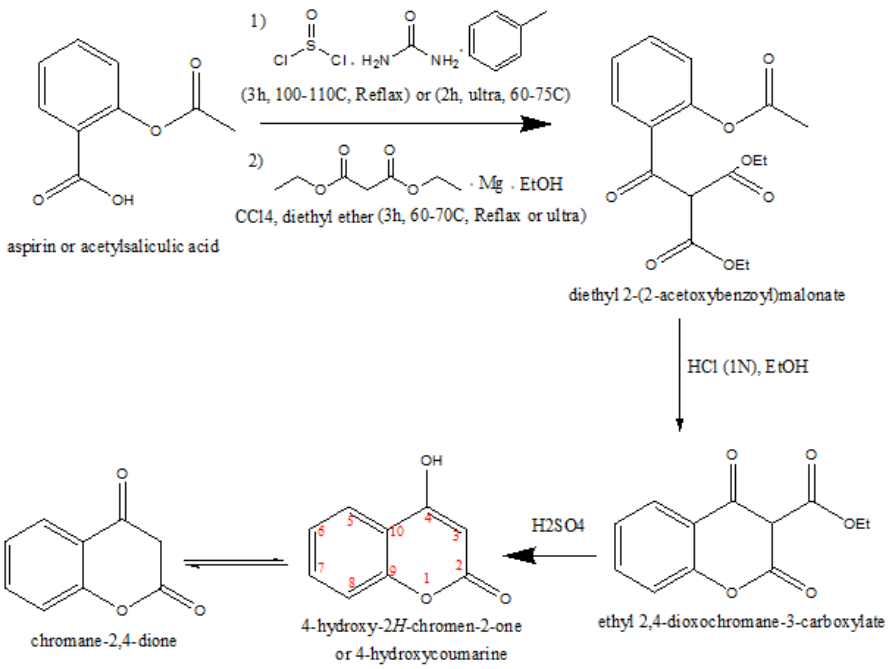


Figure 2. Reaction of aspirin and diethyl malonate in a ratio of 1: 1 in reflux / ultrasound conditions

3-Acetyl-4-hydroxycoumarin under reflux / ultrasonic bath conditions (method 2)

Figure 4 demonstrates the synthesis of 3-Acetyl-4-Hydroxycoumarin and its conversion to 4-Hydroxycoumarin under reflux and ultrasonic bath conditions. IR, ^{1}H-NMR, ^{13}C-NMR spectroscopy methods were used to identify the final structure of the products.

In IR spectroscopy of 3-acetyl-4-Hydroxycoumarin synthesized by method 2 under reflux condition, the peaks observed at 1747.77 cm^{-1} and 1670.03 cm^{-1} referred to the tensile vibration of carbonyl ester and exocyclic ketone, respectively. Furthermore, C=C tensile vibration of the aromatic ring was shown in the range of 1445.71 -1608.68 cm^{-1}. Peaks appeared at 2900-3011.33 cm^{-1} indicated the tensile vibrations of aliphatic and aromatic C-Hs. Moreover, the peak observed at 3235.96 cm^{-1} indicated OH tensile vibrations. The out of plane C-H bending vibration appeared at 758.54cm^{-1}. In ^{13}C-NMR spectroscopy, deuterium oxide solvent or D_2O was used. The peak observed in the range of 23.2 ppm indicated methyl carbon and the CH peak appeared between the two carbonyl groups (C3) at 33.45 ppm. The range of 130.4-134 ppm referred to aromatic ring carbon. The peak appeared in the range of 165.7-181.5 ppm indicated the carbons of the two carbonyl groups. In ^{1}H-NMR spectroscopy, the peak appearing at 1.83 ppm referred to solvent hydrogens and the CH3 peak of the steel group appeared at 4.72 ppm. Peaks of aromatic ring hydrogens appeared in the range of 6.8-7.8 ppm. The peak of hydrogen of the OH group is at 12.5 ppm.

Other peaks occur due to hydrolysis during the purification step or during the reaction which is some open ring product along with the main product, i.e. 3-Acetyl-4-Hydroxycoumarin (Figure 5). The peak observed at 2.108-2.442 ppm referred to CH_2 and CH_3 hydrogens and the peaks at 2.656-2.804 ppm referred to CH_2 and CH_3 hydrogens. Also, the peak observed in the range of 6.857-7.744 ppm referred to the hydrogen of the

aromatic ring while the peak appeared at 5.885 ppm referred to the hydrogen of the C-H bond. FT-IR and NMR methods were used to identify and confirm the structure of the product created using ultrasound bath. In IR spectroscopy of 3-Acetyl-4-Hydroxycoumarin synthesized by method 2 under ultrasonic bath conditions, the peaks observed at 1742.65 cm^{-1} and 1693.90 cm^{-1} referred to the tensile vibration of carbonyl ester and exocyclic ketone, respectively. The C=C tensile vibration of the aromatic ring was observed in the range of 1419.96-1608.05 cm^{-1}. Peaks appeared at 2868.32-3017.69 cm^{-1} indicated tensile vibrations of aliphatic and aromatic C-Hs. Likewise, the peak observed in 3232.49 cm^{-1} is OH tensile vibrations.

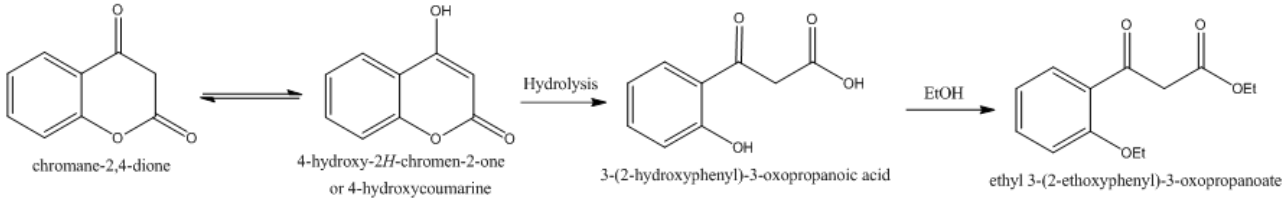


Figure 3. Open hydrolyzed form of 4-Hydroxycoumarin

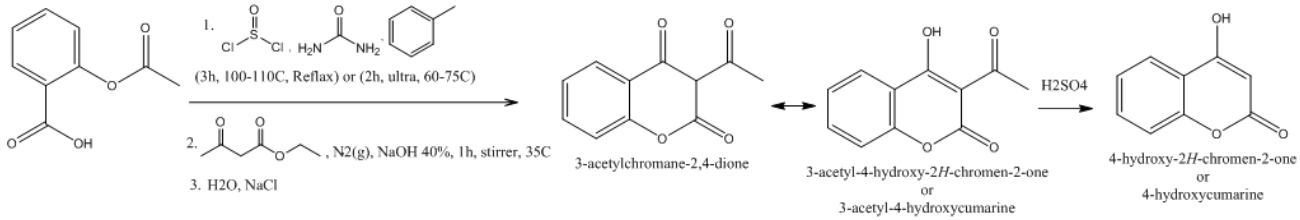


Figure 4. Synthesis reaction of 3-acetyl-4-hydroxy coumarin and its conversion to 4-hydroxy coumarin under reflux / ultrasonic bath conditions (Method 2)

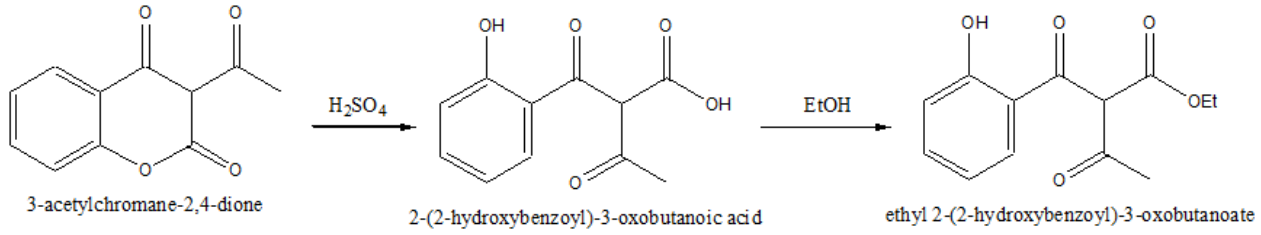


Figure 5. Open ring form with 3-Acetyl-4-Hydroxycoumarin

Hydroxycoumarin under reflux / ultrasound bath conditions (method 2)

The final structure of 4-Hydroxycoumarin synthesized by second method was analyzed by IR spectroscopy. In IR spectroscopy of 4-Hydroxycoumarin synthesized by method 2 under reflux conditions, the peak of 3236.19 cm^{-1} indicated the tensile vibrations of phenolic OH. C=O tensile vibrations were observed in 1639.10 cm^{-1}. C=C aromatic tensile vibrations appeared at 1461.63-1611.91 cm^{-1}. C-H tensile vibrations were observed at 2857.59- 3000.26 cm^{-1}. In IR spectroscopy of 4-Hydroxycoumarin synthesized by method 2 under ultrasonic bath conditions, the peak of 3614.42 cm^{-1} indicated the tensile vibrations of phenolic OH. C=O

tensile vibrations were observed in 1748.63. C=C aromatic tensile vibrations appeared in 1457.67-1620.21 cm^{-1}.

Clinical result

The effect of different doses of 4-Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin on coagulation index of relative thromboplastin time (PTT) in seconds *in vitro* is shown in Table 3. Also, Table 4 summarizes the effect of different concentrations of coumarin and 3-Acetyl-4-Hydroxycoumarin on coagulation time index (CT).

The many problems with traditional anticoagulant therapies have paved the way for the rapid spread of new drugs.

Table 1. The results of FT-IR, ^{13}C-NMR, and ^{1}H-NMR spectroscopy

Aspirin	**FT-IR:** 2994.24 cm^{-1} (carboxylic acid O–H str.), 1752.86 cm^{-1} (C=O ester str), 1686.50 cm^{-1} (C=O carboxylic acid str), 1456.84-1605.26 cm^{-1} (C=C aromatic str.).
4-hydroxy coumarin (method 1-Reflax)	**FT-IR:** 3232.49 cm^{-1} (O–H str.), 1678.41 cm^{-1} (C=O ester str), 1453.22-1605.59 cm^{-1} (C=C aromatic str.). **^{1}H-NMR (DMSO used as solvent):** 2.518 ppm (s, H of solvent), 3.27 ppm (s, H of C_3), 6.93-7.522 ppm (m, H of aromatic ring), 10.30 ppm (s, H of OH). **^{13}C-NMR (DMSO used as solvent):** 172.4 ppm (C_2), 160.6 ppm (C_4), 117.9-136.6 ppm (C_5-C_8 aromatic ring), 113.3 ppm (C_3).
4-hydroxy coumarin (method 1-Ultra)	**FT-IR:** 3235.80 cm^{-1} (O–H str.), 2862.74-3064.42 cm^{-1} (CH str), 1683.91 cm^{-1} (C=O ester str), 1483.37-1613.99 cm^{-1} (C=C aromatic str.). **^{1}H-NMR (DMSO used as solvent):** 2.53 ppm (s, H of solvent), 3.27 ppm (s, H of C_3), 6.92-7.82 ppm (m, H of aromatic ring).
4-hydroxy coumarin (method 2-Reflax)	**FT-IR:** 3236.19 cm^{-1} (O–H str.), 2857.59-3000.26 cm^{-1} (CH str), 1639.10 cm^{-1} (C=O ester str), 1461.63-1611.91 cm^{-1} (C=C aromatic str.).
4-hydroxy coumarin (method 2-Ultra)	**FT-IR:** 3614.42 cm^{-1} (O–H str.), 1748.63 cm^{-1} (C=O ester str), 1457.67-1620.21 cm^{-1} (C=C aromatic str.).
3-acetyl-4-hydroxycoumarin (method 2-Reflax)	**FT-IR:** 3235.96 cm^{-1} (OH str.), 2900-3011.33 cm^{-1} (aromatic and aliphatic CH str), 1747.77 cm^{-1} (C=O ester str), 1670.03 cm^{-1} (C=O exocyclic ketone str.), 1445.71-1608.68 cm^{-1} (C=C aromatic str.), 758.54 cm^{-1} (CH out plane bending). **^{1}H-NMR (D2O used as solvent):** 1.83 ppm (s, H of solvent), 4.72 ppm (s, H of CH_3), 6.8-7.8 ppm (m, H of aromatic ring), 12.50 ppm (s, H of OH). **^{13}C-NMR (D2O used as solvent):** 23.2 ppm (CH3), 33.4 ppm (C_3), 130.4-134 ppm (C5-C8 aromatic ring), 165.7-181.5 ppm (two carbonyl groups).
3-acetyl-4-hydroxycoumarin (method 2-Ultra)	**FT-IR:** 3232.49 cm^{-1} (OH str.), 2868.32-3017.69 cm^{-1} (aromatic and aliphatic CH str), 1742.65 cm^{-1} (C=O ester str), 1693.90 cm^{-1} (C=O exocyclic ketone str.), 1449.96-1608.05 cm^{-1} (C=C aromatic str.), 758.53 cm^{-1} (CH out plane bending).

Many patients who previously did not receive adequate treatment due to the limitations of common injectable and oral medications, can now easily be spared the risks of serious stroke and injury to cardiac arrhythmias and clots in deep veins or pulmonary arteries. Clots can block the blood flow to the heart muscle, resulting in a heart attack. The use of anticoagulants and antiplatelets in people who are at risk of blood clotting helps maintain normal blood flow. For example, heparin is the most popular anticoagulant, which is usually of natural origin. Heparin itself is a very heterogeneous mucopolysaccharide (i.e. it has different sizes). The disadvantage of heparin is that it does not have a fixed dosage due to its heterogeneity. Heparin is used when a rapid anticoagulant effect is required because it can quickly affect active factors.

Coumarins are an old group of natural family that first separated themselves from tonka beans in 1822. Coumarin and its derivatives are isolated from sweet clover, bison grass, and an insect called Woodruff. These compounds are involved in the application of plant growth hormones and growth regulators, photosynthesis, respiratory control, as well as defense against infection. Coumarins are highly regarded by researchers because of their medicinal properties. Due to their physiological properties such as anti-tumor activity, stable absorption, and screening, these compounds can be considered as new therapeutic agents. Coumarin is very important in dark pea forage plants because of the changes it makes in bacteria, it is converted to dicumarol, which is an anticoagulant and causes bleeding events in cattle. In search of phytochemicals with a wide range of biological activity and low toxicity, coumarins have attracted the attention of many research groups. Coumarins are oxygen-containing heterocycles with ordinary benzopyrene scaffolds, natural compounds isolated from various plants, fungi, and bacteria [5]. These phytochemicals have various biological and medicinal effects such as anticoagulants, antifungals, liver protectors, anti-thrombotic, antiviral, antimicrobial, anti-tuberculosis, anti-cancer, anti-depressant, anti-lipid, anti-cholinesterase, and anti-inflammatory activities [6,24]. Some coumarin-based drugs have been widely used in medicine as anticoagulants (Warfarin, Acenocoumarol, Cyclocoumarol, and Dicoumarol) and anti-neurodegenerative agents [25]. Despite the numerous biological effects described above, coumarins are considered to be one of the most versatile compounds in the design and discovery of anticancer drugs, because they provide minimal side effects along with multidrug resistance reversal activity [15]. The literature suggests that mechanisms by which coumarin and its derivatives may have anticancer activity include inhibition of telomerase or protein kinase activity, decreased oncogene expression, induction of caspase-9-mediated apoptosis, suppression of cell proliferation by cessation of G1 / G1 cell cycle G2 / M or through inhibition of P-glycoprotein in cancer cells [1,16]. Researchers select the synthesis of complexes to increase the biological activity of coumarin. In 2009, Siddiqui *et al.* discovered that coumarin is one of the applications in the epilepsy treatment and also found that anticoagulant activity at a dose of 30 mg/kg had a significant anticoagulant effect [26]. In 2013, Sripathi and Logeeswari, in a study of coumarin derivatives, found that coumarins and their derivatives are a well-known heterocyclic group which due to their wide range, they have received much attention in applications such as antimicrobial, photodynamic activity, cytotoxic properties and chemotherapy against cancer [27].

In 2014, Abdel-Wahab *et al.* found that coumarin and its derivatives were used in biology, medicine, polymer science, alcoholic beverages, pigments, laser beams, and in the production of biological compounds [28]. In 2017, Abuelizz *et al.* examined the molecular singularity and anticonvulsant activity of stabilized quinazoline derivatives [29]. In 2021, Avdovik *et al.* studied the synthesis and biological screening of new 4-hydroxy coumarin derivatives and their palladium (II) complexes [30]. Isolation, biological activity and synthesis of natural [2,3-c] coumarins are presented, which mainly covers the developments of the last 35 years. The most relevant approaches to the synthesis of 2-alternative, 3-alternative, and 2,3-alternative heterocycles are also discussed with emphasis on the efficiency of processes and their mechanisms [31]. The researchers reported the synthesis of 4H-chromene, coumarin and chromeno[2,3-d]-pyrimidine derivatives and examined the antimicrobial; antitumor activities of some of this novel series [32].

The effect of 4-hydroxycoumarin and 3-acetyl 4-hydroxycoumarin on the prothrombin time coagulation index (PT) is as follows: As shown in Table 2, 4-Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin increased both prothrombin times compared to the control group and with increasing concentration, the prothrombin time (in seconds) increased. A concentration of 10% significantly ($p < 0.05$) increased prothrombin time more than 2 orders of magnitude compared to the control group (0%) (saline 9.9%).

The effect of coumarin and 3-Acetyl-4-Hydroxycoumarin on thromboplastin relative time (PTT) coagulation index was also investigated. As can be seen in Table 3, coumarin (2, 5 and 10% by weight-volume) increased the relative thromboplastin time compared to the control group, in which 10% extract significantly $p < 0.05$ relative thromboplastin time Up to 2 times higher than the control group: recipient (0.9% saline). In the case of 3-acetyl-4-hydroxycoumarin, only 5% and 10% concentrations used had a significant effect on this coagulation index and 2% concentration with the control group (0.9% saline) did not show a significant difference (Table 4).

4- Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin both increase the coagulation time, i.e. they have an anticoagulant effect.

Table 2. The effect of different doses of 4-hydroxy coumarin and 3-acetyl-4-hydroxy coumarin on coagulation index of prothrombin time (PT) in seconds in the laboratory. Each number represents the mean ±SEM for a blood sample of five individuals repeated three times. In experiments, $p < 0.05$ was considered. Means with different letter codes have a significant difference with the control group (saline 0.9%)

Concentration of samples	Control (0.9% saline) 0%	2%	5%	10%
4- Hydroxy coumarin	$12.4^{a} \pm 2.1$	$16.1^{b} \pm 1.4$	$20.3^{c} \pm 4.6$	$24.7^{d} \pm 2.6$
3-Acetyl-4-Hydroxycoumarin	$11.9^{a} \pm 3.6$	$15.7^{b} \pm 2.2$	$20.0^{c} \pm 3.2$	$23.3^{d} \pm 2.5$

Table 3. The effect of different doses of 4-Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin on coagulation index of relative thromboplastin time (PTT) in seconds *in vitro*. Each number represents the mean ± SEM for a blood sample of five people repeated three times. P <0.05 was considered in the experiments. The means with different letter codes have a significant difference with the control group (0.9% saline)

Concentration of samples	Control(0.9% saline) 0%	2%	5%	10%
4-Hydroxy coumarin	$28.3^{a} \pm 5.1$	$45.4^{b} \pm 1.9$	$49.9^{b} \pm 4.3$	$56.7^{d} \pm 4.6$
3-Acetyl-4-Hydroxycoumarin	$32.7^{a} \pm 2.6$	$33.1^{a} \pm 2.9$	$50.1^{c} \pm 3.7$	$59.4^{d} \pm 3.5$

Table 4. Effect of different concentrations of 4-hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin on coagulation time index (CT) in minutes in vitro. Each number represents the mean ± SEM for a blood sample of five people repeated three times. P <0.05 was considered in the experiments. The means with different letter codes have a significant difference with the control group (0.9% saline)

Concentration of samples	Control (0.9% saline) 0%	2%	5%	10%
4-Hydroxy coumarin	$12.3^{a} \pm 2.1$	$22.3^{b} \pm 3.3$	$23.3^{b} \pm 2.3$	$25.4^{b} \pm 3.6$
3-Acetyl-4-Hydroxycoumarin	$14.3^{a} \pm 3.6$	$15.3^{a} \pm 2.4$	$16.2^{a} \pm 3.7$	$24.9^{b} \pm 3.5$

The effect of 4-Hydroxycoumarin was greater than that of 3-Acetyl-4-Hydroxycoumarin in terms of blood coagulation so that 4-Hydroxycoumarin showed its anticoagulant effect in all concentrations. However, 4-hydroxycoumarin had a significant anticoagulant effect at a dose of 10%.

Conclusions

Today, a large number of patients receive adequate anticoagulant treatment and prevention thanks to new anticoagulants. This greatly reduces the risk of problems such as stroke, deep vein thrombosis, and pulmonary embolism. Coumarins are a class of anticoagulants, and antiplatelet drugs that reduce the risk of blood clots in the arteries, veins or heart. The study investigated the synthesis of coumarin derivatives. It also investigated the anticoagulant effects of the products. As seen in the results section, the ultrasonic bath increased the product efficiency. It also reduced the temperature and time required to perform the reaction. Product efficiency was 57.96% in reflux method and 83.33% in ultrasound bath method. Furthermore, when using an ultrasonic bath, the product was synthesized without impurities. However, when using distillation (reflux), some open-chain product formed from the hydrolysis of a portion of 4-Hydroxycoumarin, in addition to the desired product. Temperature may have been effective in the hydrolysis of 4-hydroxycoumarin, which

has been partially hydrolyzed in the reflux method. Researchers suggest making other coumarin derivatives using both methods and comparing the results for efficiency and product type. Anticoagulant effects of products can also be investigated with the help of clinical methods. 4- Hydroxycoumarin and 3-Acetyl-4-Hydroxycoumarin both increase the coagulation time, i.e. they have an anticoagulant effect. The effect of 4-Hydroxycoumarin was greater than that of 3-Acetyl-4-Hydroxycoumarin in terms of blood coagulation so that 4-Hydroxycoumarin showed its anticoagulant effect in all concentrations. Chemical synthesis and structural modifications of 4-Hydroxycoumarin and its derivatives are of interest due to their biological activity and characteristic conjugated molecular architecture. It is suggested that new and different approaches to the synthesis of 4-Hydroxycoumarin derivatives and research on their biological activities be explored in studies.

Orcid

Sabah Salahvarzi : 0000-0003-0965-0723

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