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ORIGINAL ARTICLE

Antioxidant Properties of 5,7-Dihydroxycoumarin Derivatives in *in vitro* Cell-free and Cell-containing Systems

Mei-Hsiang Lin [1], Yu-Shiang Chou [2], Yan-Jyu Tsai [3], Duen-Suey Chou [3]*

[1] School of Pharmacy, College of Pharmacy, Taipei Medical University, Taipei, Taiwan
[2] Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan
[3] Department of Pharmacology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

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Background: Coumarin is a well-known plant-derived natural product, which is extensively used as a biologically active compound. Coumarins that possess hydroxyl groups in their benzenoid ring were shown to have potent antioxidant and radical-scavenging properties in various experimental models.
Purposes: During our search for new types of coumarin derivatives possessing antioxidant activity, we investigated the synthesis of 5,7-dihydroxycoumarin derivatives and attempted to find potential therapeutic candidates to treat oxidative stress.
Methods: A series of different cell-free and cell-containing tests was conducted. In cell-free systems, these compounds were tested by means of three electron spin resonance assays [a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, a xanthine/xanthine oxidase system, and a hydrogen peroxide/sodium hydroxide/dimethyl sulfoxide]. In cell-containing systems, baicalein-induced hydroxyl radical formation by B16F10 cells was used to evaluate the antioxidant properties of the coumarins. Finally, we measured the protective capacity of these coumarins against oxidative damage caused by baicalein-induced cytotoxicity in B16F10 cells.
Results: All of the tested compounds were potent DPPH and superoxide radical scavengers in the DPPH and xanthine/xanthine oxidase systems, respectively. In the hydrogen peroxide/sodium hydroxide/dimethyl sulfoxide system, Compound **4** was the best radical scavenger among the 11 compounds tested. Compounds **6** and **8** were also potent hydroxyl radical scavengers but increased the formation of methyl radicals. We further evaluated the effects of the test compounds on living cells, and only Compounds **1**, **3**, **5**, and **6** inhibited baicalein-induced hydroxyl radical formation in B16F10 cells. However, none of them could reduce baicalein-induced cytotoxicity in B16F10 cells.
Conclusions: The different methods used to assess the antioxidant activities of the tested compounds can give varying results depending on the specific free radical and solvent system being used. Certain 5,7-dihydroxy-coumarins possess high antioxidant activity *in vitro*. Baicalein-induced cytotoxicity in B16F10 cells did not seem to be completely related to ROS generation.

1. Introduction

Reactive oxygen species (ROS) are formed in living cells via both enzymatic and nonenzymatic mechanisms. Some ROS are required for the creation of specific physiological functions, and some ROS formation is involved in the pathogenesis of a number of diseases.[1] It is well documented that antioxidants can decrease the risk of age-related human diseases.[2,3] Antioxidants may react with ROS and decrease their toxic actions, and for that reason, many scientists are searching for novel natural and synthetic antioxidants.

Coumarin agents (known as 1,2-benzopyrone), consisting of fused benzene and α-pyrone rings, are present in significant amounts in plants, and more than 1300 coumarins were identified from natural sources.[4] These natural compounds serve as important models for advanced design and synthesis of more active analogues. Natural and synthetic coumarins were verified to have antioxidant, anti-inflammatory, anticoagulation, estrogenic, dermal photosensitizing, vasodilator, molluscicidal, antihelmintic, sedative, hypnotic, analgesic, hypothermic, and antiulcer activities.[5,6] The antioxidant effects were widely investigated. Polyhydroxy (phenolic) coumarins are known to act as antioxidants in biological systems. The *O*-dihydroxy- and *O*-diacetoxy-substituted coumarins were demonstrated to be excellent radical scavengers.[7,8] In addition, dihydroxy and diacetoxy derivatives of thionocoumarin showed more potent antioxidant effects than the corresponding coumarins.[9]

* Corresponding author. Department of Pharmacology, School of Medicine, College of Medicine, Taipei Medical University, 250 Wuxing Street, Taipei 11031, Taiwan.
E-mail: D.-S. Chou <fird@tmu.edu.tw>

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Electron spin resonance (ESR) is a spectroscopic technique that can directly detect free radicals and is commonly applied to evaluate the free radical-scavenging activity of antioxidants in both cell-free and cell-containing systems. In this study, we determined the radical-scavenging capacity of 11 structurally related synthetic 5,7-dihydroxy-coumarins by measuring their reaction with radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anions, and in the hydrogen peroxide/sodium hydroxide/dimethyl sulfoxide (H_2O_2/NaOH/DMSO) system using ESR spectroscopy. To explore clinical applications of these free radical-scavenging compounds, we also examined their antioxidant activities in a living-cell system.

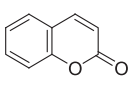
2. Materials and Methods

2.1. Chemicals and reagents

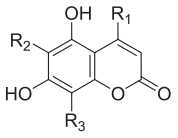
The coumarin derivatives used in this study were synthesized in our laboratory and published previously (Chart 1).[10] The following coumarins (their structure numbers and code names are given in parentheses) were dissolved in DMSO: serratin (Compound **1**); 5,7-dihydroxy-6-(3-methyl-butyryl)-4-phenyl-chromen-2-one (Compound **2**); 5,7-dihydroxy-6-(3-methyl-butyryl)-4-methyl-chromen-2-one (Compound **3**); 5,7-dihydroxy-6-(3-methyl-butyryl)-4-ethyl-chromen-2-one (Compound **4**); 6-benzoyl-5,7-dihydroxy-4-phenyl-chromen-2-one (Compound **5**); 6-(phenylacetoyl)-5,7-dihydroxy-4-phenyl-chromen-2-one (Compound **6**); 5,7-dihydroxy-6-propyryl-4-ethyl-chromen-2-one (Compound 7); 5,7-dihydroxy-6-acetoyl-4-ethyl-chromen-2-one (Compound **8**); 6-(3,3-dimethyl-butyryl)-5,7-dihydroxy-4-ethylchromen-2-one (Compound **9**); 6-(2-cyclohexanoyl)-5,7-dihydroxy-4-ethyl-chromen-2-one (Compound **10**); and 5,7-dihydroxy-6-(3-methyl-butyryl)-4-(p-methylphenyl)-chromen-2-one (Compound **11**). Baicalein; DMSO; 5,5-dimethyl *N*-oxide pyrroline (DMPO); iron (II) sulfate heptahydrate; 30% H_2O_2; tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); xanthine (X); xanthine oxidase (XO); allopurinol; and DPPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amphotericin B (Fungizone), Dulbecco's modified Eagle medium (DMEM), porcine elastase, fetal calf serum (FCS), L-glutamine, penicillin/streptomycin, sodium pyruvate, trypan blue stain, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals and reagents were of the highest commercial grade and were used without further purification.

2.2. Tumor cell lines and cell culture

B16F10 murine melanoma cells were obtained from the National Institute of Preventive Medicine, Department of Health, Executive Yuan (Taipei, Taiwan) and were cultured at 37°C, under a humid atmosphere and 5% CO_2, in Rosewell Park Memorial Institute-1640 medium with 10 mM HEPES, 24 mM sodium bicarbonate, 40 mg/L gentamycin (pH 7.2), and 10% FCS.



coumarin



1. R_1 = Ph, R_2 = H, R_3 = H
2. R_1 = Ph, R_2 = $COCH_2CH(CH_3)_2$, R_3 = H
3. R_1 = CH_3, R_2 = $COCH_2CH(CH_3)_2$, R_3 = H
4. R_1 = CH_2CH_3, R_2 = $COCH_2CH(CH_3)_2$, R_3 = H
5. R_1 = Ph, R_2 = COPh, R_3 = H
6. R_1 = CH_2CH_3, R_2 = $COCH_2Ph$, R_3 = H
7. R_1 = CH_2CH_3, R_2 = $COCH_2CH_3$,R_3 = H
8. R_1 = CH_2CH_3, R_2 = $COCH_3$, R_3 = H
9. R_1 = CH_2CH_3 , R_2 = $COCH_2C(CH_3)_3$, R_3 = H
10. R_1 = CH_2CH_3, R_2 = COcyclohexyl, R_3 = H
11. R_1 = *p*-CH_3, R_2 = $COCH_2CH(CH_3)_2$, R_3 = H

Chart 1. Structures of 5,7-dihydroxycoumarins.

2.3. Proliferation assay

Cell viability was assessed using a standard MTT assay. B16F10 cells in the exponential growth phase were suspended in DMEM containing 10% FCS and cultured in flat-bottomed, 96-well plates (2×10^4 cells/well) for 24 hours at 37°C, then baicalein was added. The final concentrations of the drug were 10 μM, 25 μM, and 50 μM, and plain DMEM was used as the negative control. Plates were incubated in a humidified incubator of 5% CO_2 at 37°C for 6 hours, 12 hours, 18 hours, and 24 hours. Supernatants were discarded after centrifugation. MTT (0.5 mg/mL) at 100 μL/well was added to the plates, and incubation continued for 3 hours. The supernatant was then carefully removed, and 300 μL of DMSO was added to dissolve the formazan crystals. The optical density at 540 nm was read using an enzyme-linked immunosorbent assay reader. The percentage of cell viability was calculated as the absorbance of treated cells/control cells × 100%.

2.4. DPPH radical-scavenging activity

Activities of scavenging DPPH, a stable radical, by the coumarins were estimated according to the laboratory method described by Chang et al[11] with minor modifications. Stock solutions of coumarins and DPPH were prepared in methanol. Reaction mixtures contained 100 μM of coumarins and 0.1 mM DPPH. The ESR signal was recorded 90 minutes after mixing the sample and reagent under the following instrument settings: microwave power of 20 mW, field modulation of 1.0 G, and a scan range of 100 G. The percentage of DPPH scavenged was estimated using a solvent-treated sample as the control.

2.5. Superoxide radical anion-scavenging activity

DMPO was used to trap and detect superoxide radical anions in the measuring reaction mixture by ESR spectroscopy. The X-XO system was used to generate radical anions. Final reaction mixtures contained 1.5 mM X, 100 mM DMPO, 0.1 mM DTPA, 0.12 U/mL XO, and 20 μM of coumarins in phosphate-buffered saline. The radical-scavenging reaction was initiated by the addition of XO, and the ESR signal was recorded 1 minute later. ESR spectra were obtained at room temperature under the following instrument conditions: microwave power of 20 mW, field modulation of 1 G, and a scan range of 100 G.

2.6. ESR spin trapping assay: the H_2O_2/NaOH/DMSO system

The ESR spectrum was obtained in the H_2O_2/NaOH/DMSO system as described previously[12] with minor modifications. Briefly, 50 μL of DMSO and the same volumes of 25 mM NaOH and sample solution (aqueous) were mixed in a test tube, followed by the addition of 1.5 μL of DMPO and 50 μL of 30% H_2O_2. The reaction mixture was aspirated into a quartz flat cell and set in the ESR apparatus; scanning began 1 minute after all reagents were mixed. The positive signal height of methyl radical-DMPO adducts and hydroxyl radical-DMPO adducts and the negative signal height of superoxide anion-DMPO adducts were calculated in the lowest magnetic field.

2.7. Baicalein-induced hydroxyl radical-scavenging activity

Scavenging activities by the coumarins of baicalein-induced hydroxyl radicals were estimated according to our previous

study[13] with some modifications. In brief, a B16F10 melanoma cell suspension (5×10^5 cells/150 μL) was preincubated with DMPO (100 mM), and then the coumarins (20 μM) were added for 3 minutes before the addition of baicalein. The dead time of sample preparation and ESR analysis was exactly 60 seconds after the last addition. Conditions of ESR spectrometry were as follows: 20 mW of power at 9.78 GHz, with a scan range of 100 G and a receiver gain of 5×10^4. The modulation amplitude, sweep time, and time constant are given in the legends to the figures and tables.

2.8. Statistical analysis

Experimental results are expressed as the mean ± standard error of the mean (SEM) and are accompanied by the number (n) of observations. Data were assessed using an analysis of variance. If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. DPPH radical-scavenging activity

The model of scavenging the stable radical, DPPH, is a widely used method to evaluate antioxidant capacities of natural and synthetic products. To determine whether the lipophilic coumarins could directly interact with and scavenge DPPH radicals, the reactions of 11 samples with DPPH radicals were tested at 200 μM using ESR. Typical ESR spectra are shown in Figure 1A. Signal intensities were reduced upon the addition of any of the 11 samples. The relative scavenging abilities are shown in Figure 1B. Compounds **6**, **10**, and **11** were the most potent agents in reducing DPPH radicals among the coumarins tested. Other selected coumarins displayed less potent effects in scavenging DPPH radicals. These results demonstrated that the coumarins tested possess the ability to scavenge the DPPH radical.

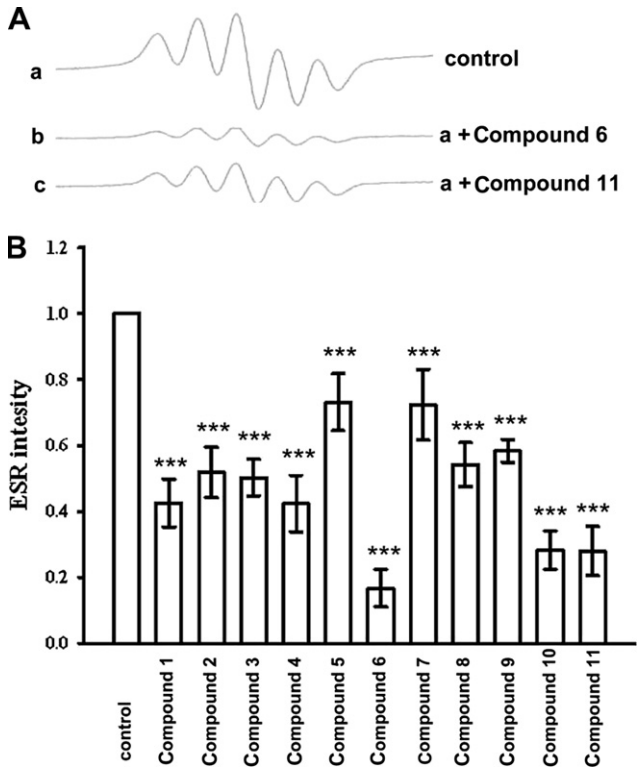


Figure 1 Antioxidative activities of coumarins against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. (A) Typical electron spin resonance spectra obtained from the reaction of DPPH followed by the addition of (a) dimethyl sulfoxide (control), (b) 100 μM of Compound **6**, and (c) 100 μM of Compound **11**. (B) Effects of 100 μM of the coumarin derivatives on the intensity of the DPPH control solution. Data are shown as the mean ± standard error of the mean of five independent experiments. $^{***}p < 0.001$, compared with the control. The instrument parameters were as follows: a modulation amplitude of 1 G, a time constant of 164 milliseconds, and scanning for 40.96 milliseconds with accumulation of six scans.

3.2. Superoxide radical anion-scavenging activity

The X-XO reaction is an important biological source to generate superoxide radical anions, and this reaction is known to be involved in numerous pathological processes. Superoxide anions can inhibit the citric acid cycle enzyme, aconitase, at very low concentrations.[14] We used ESR combined with the DMPO trapping assay and X/hypoxanthine in the presence or absence of coumarins to evaluate their total activities for ROS scavenging and reducing ROS formation. In the hypoxanthine/XO system, all 11 samples suppressed the signal of the superoxide anion-DMPO adduct on the ESR charts (Figure 2A). The relative scavenging ability is shown in

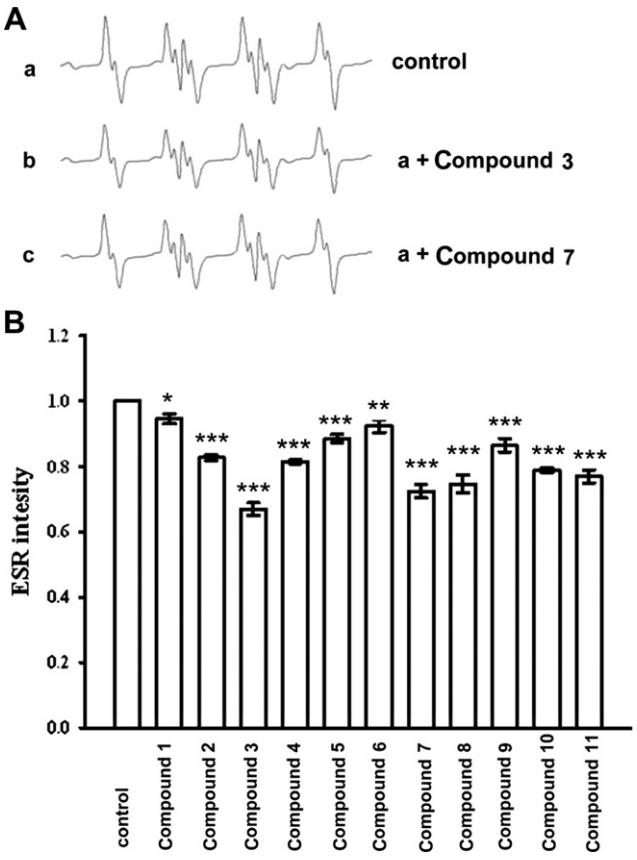


Figure 2 Antioxidative activities of coumarins against the superoxide radical. (A) Typical electron spin resonance spectra obtained from the reaction of xanthine oxidase/hypoxanthine in the presence of 5,5-dimethyl *N*-oxide pyrroline (DMPO) (100 mM) followed by the addition of (a) dimethyl sulfoxide (DMSO) (control), (b) 20 μM of Compound **3**, and (c) 20 μM of Compound **7**. (B) Effects of 20 μM of the coumarin derivatives on the intensity of xanthine oxidase/hypoxanthine-induced superoxide generation. Xanthine oxidase/hypoxanthine was preincubated with DMPO (100 mM) followed by the addition of DMSO (control). The data are shown as the mean ± standard error of the mean of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control. The instrument parameters were exactly the same as those in Figure 1.

Figure 2B. Compound **3** was the most potent agent in reducing superoxide anion radicals among the coumarins tested. Because the X/XO system is based on an enzymatic reaction, nonspecific interactions with the protein cannot be excluded from the antioxidant mechanisms of the test compounds.

3.3. Radical-scavenging activity in the H_2O_2/NaOH/DMSO system

Superoxide anions, hydroxyl radicals, and methyl radicals are simultaneously generated by the H_2O_2/NaOH/DMSO system. This system is a nonenzymatic and non-Fenton type of ROS-generating system. When all of the components were mixed, typical spectra of superoxide anions, hydroxyl radicals, and methyl radicals were visible as shown in Figure 3A. In this system, Compound **4** significantly suppressed the formation of all kinds of free radicals, such as methyl radicals, hydroxyl radicals, and superoxide anions (Figure 3D). As to the hydroxyl radical-scavenging activity, Compound **8** was the most potent agent among the coumarins tested (Figure 3G). L-Ascorbic acid totally suppressed the formation of hydroxyl radicals and superoxide anions but increased the signal height of methyl radicals (Figure 3H). In addition, Compounds **2** and **3** displayed less potent effects (Figure 3B and C), and other selected coumarins had no significant effect of radical scavenging (data not shown).

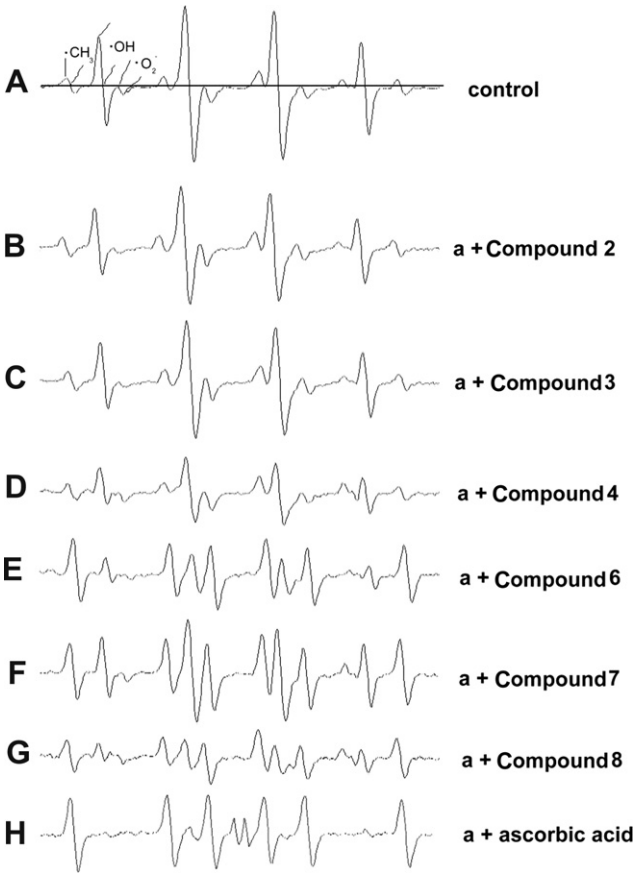


Figure 3 Free radical-scavenging activities of coumarins in the hydrogen peroxide/sodium hydroxide/dimethyl sulfoxide (H_2O_2/NaOH/DMSO) system. (A) Each free radical-derived electron spin resonance (ESR) signal was assigned in the control. Typical ESR spectra obtained by the addition of (B) 200 μM of Compound **2**, (C) 200 μM of Compound **3**, (D) 200 μM of Compound **4**, (E) 200 μM of Compound **6**, (F) 200 μM of Compound **7**, (G) 200 μM of Compound **8**, and (H) 200 μM of ascorbic acid in the H_2O_2/NaOH/DMSO system. Conditions of ESR spectrometry were as follows: a power of 20 mW, a modulation frequency of 100 kHz, a modulation amplitude of 1 G, a receiver gain of 6.3×10^{-4}, a time constant of 164 milliseconds, and a conversion time of 40.96 milliseconds. The spectrum is a representative example of four similar experiments.

3.4. Coumarin derivatives hydroxyl radical formation in B16F10 cells

Our previous study demonstrated that baicalein induces inhibition of proliferation in B16F10 melanoma cells by generating ROS via 12-lipoxygenase.[13] We used ESR combined with the DMPO trapping assay in this cell-containing system in the presence and absence of the 11 coumarins to evaluate their total activities for ROS scavenging and reducing ROS formation. Their relative scavenging abilities are shown in Figure 4. The typical ESR spectrum for the spin adduct between hydroxyl radicals and the spin trapping DMPO is shown in Figure 4A. Only Compounds **1**, **3**, **5**, and **6** (at 20 μM) significantly reduced the signal intensity of the spin adduct. Other selected coumarins had no significant effect on ROS scavenging or reducing ROS formation.

3.5. Coumarin derivatives baicalein-induced cytotoxicity in living cells

Because the above results showed that coumarins are effective agents in reducing oxidative stress, we further evaluated the effects

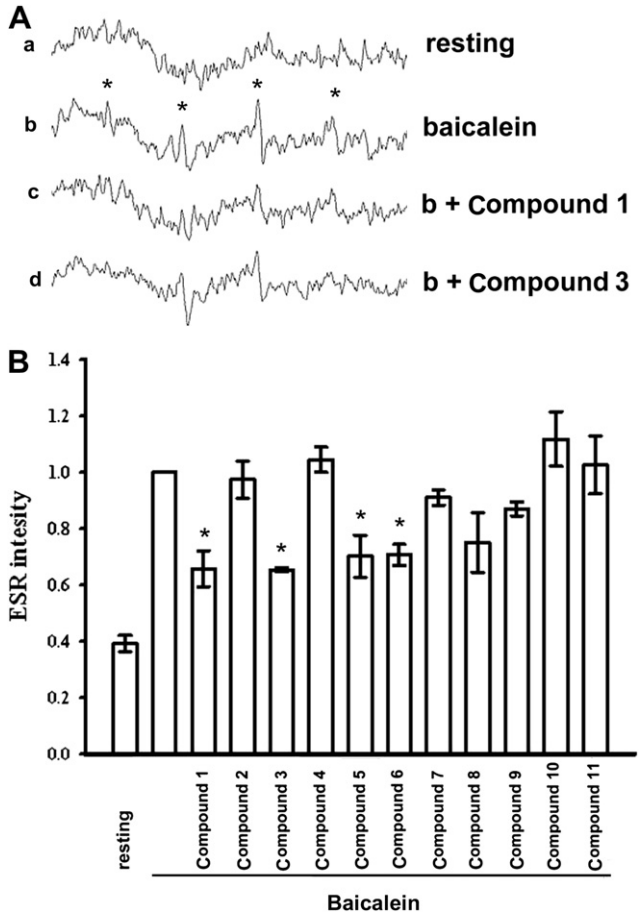


Figure 4 Free radical-scavenging activities of coumarins in the baicalein/B16F10 cell system. (A) Electron spin resonance (ESR) spectra obtained from the reaction of B16F10 melanoma cells (5×10^5 cells/150 μL) in the presence of DMPO. B16F10 melanoma cells were preincubated with 5,5-dimethyl *N*-oxide pyrroline (DMPO) (100 mM) followed by the addition of (a) dimethyl sulfoxide (solvent control), (b) 50 μM of baicalein, (c) 20 μM of Compound **1**, and (d) 20 μM of Compound **3**. (B) Effects of 20 μM of the coumarin derivatives on the intensity of the baicalein-induced hydroxyl radical signal. Data are shown as the mean ± standard error of the mean of five independent experiments. $^*p < 0.05$, compared with baicalein treatment only. The instrument parameters were as follows: a modulation amplitude of 1 G, a time constant of 164 milliseconds, and scanning for 40.96 milliseconds with 12 scans accumulated. The ESR spectra are labeled to show their components of the DMPO-hydroxyl radical adduct (*).

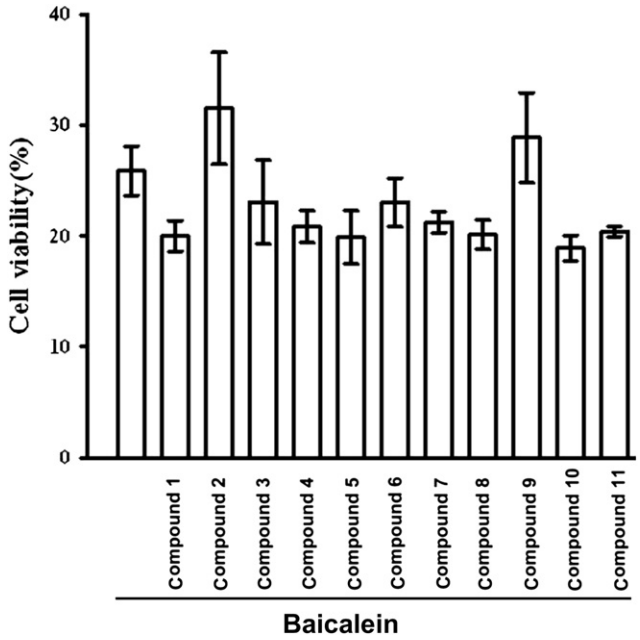


Figure 5 Inhibition of baicalein-induced cytotoxicity by coumarin derivatives in living cells. B16F10 melanoma cells (10^6 cells/mL) were dispensed on 96-well plates. Cells were treated with the indicated concentration of 20 μM of the coumarin derivatives for 1 hour before treatment with 50 μM baicalein for 24 hours. Cell viability was measured by the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. B16F10 cells without baicalein treatment served as a control. The percentage viability is presented as the mean ± standard error of the mean of three independent experiments.

of coumarin derivatives in rescuing B16F10 from oxidative stress. B16F10 cells were treated with baicalein (50 μM) for 24 hours in the presence and absence of the 11 coumarins, and the results of cell viability were determined by measuring the metabolism of the tetrazolium substrate, MTT, as shown in Figure 5. Cell viability after baicalein treatment for 24 hours was about 26.4%, which was rescued by Compounds **2** and **9** (20 μM) to about 29.1% and 31.2%, respectively. The protective effects were calculated according to the reduced cytotoxicity with coumarin treatment (20 μM). This result showed no obvious significance, possibly because the solubility of coumarins in the culture medium meant that we were unable to use higher concentrations. Activities of coumarins against the baicalein-induced reactive HO• have not been extensively studied; however, Compounds **2** and **9** were more potent than vitamin C at a concentration of 20 μM in this system (data not shown).

4. Discussion

Methods of assessing antioxidant activity using free radical traps are relatively simple to perform. A plain method that was developed to determine the antioxidant activity of tested compounds utilizes the stable DPPH radical. This is a widely used method for determining the ability of a test compound to scavenge free radicals through donation of a hydrogen atom or electron transfer.[15] The DPPH method can be used for solid or liquid compounds and is not specific to any particular antioxidant component. This DPPH-electron paramagnetic resonance system allows direct comparison of the radical-scavenging activities of hydrophilic and lipophilic antioxidants under similar conditions.[16]

The pharmacological properties of simple coumarins depend upon the model of substitution. A series of 11 coumarins was tested in this study in several systems involving ROS to characterize their antioxidant potential. We identified that all of our coumarins possess the ability to scavenge free radicals in the DPPH and X/XO systems. The radical-scavenging effects of coumarins are correlated with the number of hydroxyl groups on the benzene rings.[17,18] The chemical structures of our tested coumarins bore two hydroxyl moieties on the benzene rings, and the results of our study are consistent with those of previous studies.

XO is an important source of free radical generation in post-ischemic cells and tissues,[19] and the X/XO system is based on an enzymatic reaction. The problem with this assay is that it is not suitable for lipophilic antioxidants because of their poor water solubility, and it is not clear whether the antioxidants directly react with the radical or suppress XO activity. Chang and Chiang[20] demonstrated that the structure of 7-hydroxy coumarin plays a very important role in XO inhibition. Our test samples in this study were 5,7-dihydroxy coumarins, and it is not surprising that all 11 samples could significantly suppress signals generated by the X/XO system at a low concentration (20 μM) (Figure 2).

Antioxidant compounds may be water-soluble, lipid-soluble, or lipid-insoluble. Hence, the assay solvent system is an important factor in quantifying the antioxidant activities of test compounds. Lipid-soluble compounds cannot be measured using aqueous-based assays. Much effort was expended to improve the solubility of lipophilic antioxidants in aqueous systems.[21,22] H_2O_2/NaOH/DMSO was developed to produce three free radicals.[12] Both water- and oil-soluble antioxidants can be evaluated using this system. In this system, the methyl radical is formed by the reaction of ascorbic acid with free radicals to generate ascorbyl radicals, which attacks DMSO and becomes the methyl radical, or the ascorbyl radical is directly degraded into the methyl radical.[12] Compounds **6** and **8** also increased the formation of methyl radicals (Figure 3E–G). Ascorbic acid exhibits prooxidant properties, and the ascorbyl radical might cause oxidative reactions with other compounds.[23] However, Compound **4** did not form its own radical (Figure 3D), and thus, we suggest that it is a better radical scavenger then Compounds **6**, 7, or **8**.

The different methods used to assess the antioxidant activities of the test compounds can produce varying results depending on the specific free radical being used as the reactant. In addition, the antioxidant activity of phenols depends on the electronic and steric effects of the ring, substituents, and strength of hydrogen-bonding interactions between the phenol and solvent.[24] In this study, we used different free radicals and solvent systems to assess the antioxidant activities of the tested coumarins. It is no wonder that the tested coumarins showed different antioxidant activities in each assay.

Intracellular ROS production is associated with a number of cellular events and is involved in the pathogenesis of several diseases.[1] However, antioxidants have not proven to be valuable against many of these diseases. One possible reason is that some of the antioxidants do not arrive at the appropriate sites of free radical generation. Another possibility is that many of the antioxidants, such as baicalein, can also have prooxidant activities in addition to their antioxidant activities. We thus examined the antioxidant effects of synthetically obtained 5,7-dihydroxy coumarins on oxidative stress in a living cell system. B16F10 melanoma cells were chosen in this assay system, and oxidative stress was created by adding baicalein according to our previous study.[13] The results of this study demonstrated that Compounds **1**, **3**, **5**, and **6** reduced baicalein-induced reactive HO•, but all of the tested coumarins had a negligible effect on baicalein-induced cytotoxicity in B16F10 cells. This result suggests that baicalein-induced cytotoxicity does not totally depend on ROS formation. This is consistent with observations of previous studies, which showed that baicalein could inhibit proliferation of various tumor cell lines, such as human myeloma RPMI8226 cells[25] and H460 human lung nonsmall carcinoma cells,[26] by inhibiting 12-LOX protein expression. In addition, baicalein downregulates 12-LOX expression possibly by diminishing activity of the transcription factor, nuclear factor-κB, in human myeloma cells.[27]

From these results, it was also possible to make a number of correlations regarding the relationship between the structure of the coumarins and their antioxidative activities.

- As far as DPPH radical-scavenging activity, six acyl substituents of 4-phenyl-5,7-dihydroxycoumarins showed decreased bioactivity (**1** ($R_2 = H$) > **2** ($R_2 = COCH_2CH(CH_3)_2$) > **5** ($R_2 = COph$)). 6-(3-Methyl-butyryl)-5,7-dihydroxycoumarins which were substituted in 4-phenyl group showed enhanced bioactivity (**11** ($R_1 = C_6H_4(p\text{-}CH_3)$) > **2** ($R_1 = ph$)). It was found that the antioxidative activities of 6-acyl-4-aryl-5,7-dihydroxycoumarins decreased in the following sequence: **11** > **1** > **2** > **5** (i.e., 6-(3-methyl-butyryl)-4-(*p*-methylphenyl) > 4-phenyl > 6-(3-methyl-butyryl)-4-(phenyl) > 6-benzoyl-4-phenyl). Among 4-alkyl-6-(3-methyl-butyryl)-5,7-dihydroxycoumarins, 4-ethyl had better activity than 4-methyl (**4** > **3**). Among 4-ethyl-6-acyl-5,7-dihydroxycoumarins, long-chain and bulky substituents were more active than were small-chain ones (**6** ($R_2 = COCH_2ph$) > **10** ($R_2 = COcyclohexyl$) > **4** ($R_2 = COCH_2CH(CH_3)_2$) > **8** ($R_2 = COCH_3$) > **9** ($R_2 = COCH_2C(CH_3)_3$) > **7** ($R_2 = COCH_2CH_3$)). This suggests that introducing a 6-phenylacetoxyl group to 4-phenylcoumarin has a positive influence on its antioxidative activity.
- As to the superoxide radical anion-scavenging activity, six acyl substituents of 4-phenyl-5,7-dihydroxycoumarins showed increased bioactivity (**2** ($R_2 = COCH_2CH(CH_3)_2$) > **5** ($R_2 = COph$) > **1** ($R_2 = H$)). Among 4-alkyl/aryl-6-(3-methyl-butyryl)-5,7-dihydroxycoumarins, 4-methyl had better activity than 4-ethyl/aryl (**3** ($R_1 = CH_3$) > **4** ($R_1 = CH_2CH_3$) > **11**($R_1 = C_6H_4(p\text{-}CH_3)$). Among 4-ethyl-6-acyl-5,7-dihydroxycoumarins, short-chain substituents were more active than long-chain ones (**7** ($R_2 = COCH_2CH_3$), **8** ($R_2 = COCH_3$) > **4** ($R_2 = COCH_2CH(CH_3)_2$) > **9** ($R_2 = COCH_2C(CH_3)_3$) > **6** ($R_2 = COCH_2ph$)). In this assay, 4-methyl substitution of 4-alkyl/arylcoumarins showed increased bioactivity.
- As to the radical-scavenging activity in the H_2O_2/NaOH/DMSO system assay, among 4-alkyl/aryl-6-(3-methyl-butyryl)-5,7-dihydroxycoumarins, 4-ethyl had better activity than 4-methyl/aryl (**4** ($R_1 = CH_2CH_3$) > **2** ($R_1 = ph$), **3** ($R_1 = CH_3$)). Among 4-ethyl-6-acyl-5,7-dihydroxycoumarins, short-chain substituents were more active than long-chain ones (**8** ($R_2 = COCH_3$) > **4** ($R_2 = COCH_2CH(CH_3)_2$) > **6** ($R_2 = COCH_2ph$)). This suggests that introducing a 6-acetoxyl group to 4-alkyl/phenylcoumarin has a positive influence on its antioxidative activity.
- In the coumarin-inhibited baicalein-induced hydroxyl radical formation assay in B16F10 cells, the antioxidative activity of 6-acyl-4-phenyl-5,7-dihydroxycoumarins decreased in the following sequence: **1** ($R_2 = H$), **5** ($R_2 = COph$) > **2** ($R_2 = COCH_2CH(CH_3)_2$). A 4-phenyl group substitution in 6-(3-methyl-butyryl)-5,7-dihydroxycoumarins had no effect on their activities (**11** ($R_1 = C_6H_4(p\text{-}CH_3)$) = **2** ($R_1 = ph$)). Among 4-alkyl-6-(3-methyl-butyryl)-5,7-dihydroxycoumarins, 4-methyl had better activity than 4-ethyl (**3** > **4**). Among 4-ethyl-6-acyl-5,7-dihydroxycoumarins, the phenylacetoxyl substituent was more active than the others (**6** ($R_2 = COCH_2ph$) > **7** ($R_2 = COCH_2CH_3$), **8** ($R_2 = COCH_3$), **9** ($R_2 = COCH_2C(CH_3)_3$) > **4** ($R_2 = COCH_2CH(CH_3)_2$), **10** ($R_2 = COcyclohexyl$)).

In conclusion, the present study showed that certain 5,7-dihydroxy-coumarin derivatives possess *in vitro*. All of them are DPPH and superoxide radical scavengers. In the H_2O_2/NaOH/DMSO system, Compound **4** was the best radical scavenger among the 11 tested compounds. Compounds **6** and **8** were also potent hydroxyl radical scavengers but increased the formation of methyl radicals. In living cells, the protective activities of the test compounds against baicalein-induced cytotoxicity did not seem to be related to their HO•-scavenging or -reducing activities. These compounds may be helpful in designing new therapeutic tools against oxidative stress.

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