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Research paper

Antioxidant and antitumor activities of 4-arylcoumarins and 4-aryl-3,4-dihydrocoumarins

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ABSTRACT

Five 4-arylcoumarins (**1c**–**g**) and twelve 3,4-dihydro-4-arylcoumarins (**2a**–**l**) were synthesized and tested for antioxidant activity, antitumor activity, toxicity and structure–activity relationships analysis. 4-Arylcoumarins and 3,4-dihydro-4-arylcoumarins that possess two hydroxyl groups in *ortho* position, such as **1d**, **1f**, **2a**, **2f**, **2g** and **2h** had stronger radical scavenging properties than that of vitamin C (Vit C) in $ABTS^{\bullet+}$ assay. Kinetic traces of scavenging $ABTS^{\bullet+}$ and DPPH radicals showed that all the reaction could reached endpoint in 1 min, which was similar with Vit C. 4-Arylcoumarins with 3′-hydroxyl-4′-methylphenyl structural show more efficient $NO^{\bullet}$ radical scavenging activity. Three compounds **2e**, **1f** and **2a**, in particular had superior EC_{50} for $NO^{\bullet}$ scavenging than did Vit C. MTT assay indicated that one compound in particular had a potential antitumor effect, inhibiting proliferation of BGC-823 cells and almost completely killing them at a concentration 62.5 mg/L. With same concentration 100 μg/mL, hemolytic analysis in rabbit red blood cells showed that only two compounds had hemolytic activity with a little more than 5% hemolysis. Injection and oral toxicity tests on *Galleria mellonella* larvae showed that none of the tested 4-arylcoumarins significantly affected their appetite, viability and mortality.

1. Introduction

Coumarins are found naturally in many green plants and natural food products, and have been widely used as an aroma enhancer in foods and pastries [1]. 4-Arylcoumarins and 3,4-dihydro-4-arylcoumarins represent a minor class of natural coumarin derivatives commonly found in fruit and vegetables, with a C6–C3–C6 skeleton and a characteristic 4-aryl group. More than 130 natural 4-arylcoumarins and 3,4-dihydro-4-arylcoumarins have been isolated from plants of the Clusiaceae, Fabaceae, Rubiaceae, Asteaceae, Thelypteridaceae, Passifloraceae and Rutaceae families [2–4]. Both natural and synthetic 4-arylcoumarins have exhibited antioxidant [5–7], anticancer [8,9], antimicrobial [10,11], and anti-inflammatory properties [12].

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. Low concentrations of free radicals play very important roles in various physiological functions, such as signal transduction, vessel pressure control, immune response among others [13]. Over production of free radicals results in oxidative stress that can be an important source of damage to cell structures, such as cell membranes, proteins, and DNA [14]. As reviewed, heart disease [15], central nervous system disorders [16], aging process and cancer development [17] may be caused by free radical over-produced. We have reported previously that several synthetic 4-arylcoumarins and 3,4-dihydro-4-arylcoumarins with two hydroxyl groups placed *ortho* to each other in the benzene ring exhibited significantly stronger DPPH free radical scavenging properties than traditional antioxidant reagents, such as Vit C and butylated hydroxytoluene, which indicated that these 4-arylcoumarin derivatives may have potential in as biomedicines [6]. However, the scavenging ability to other free radicals, the biosecurity to animals and the relationship between structural and antioxidant activities of those coumarins remains unclear. Meanwhile, a number of researchers have reported that some coumarins are toxic to insects, such as furanocoumarins which have appetite

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suppress in ant and growth inhibition activities in *Depressaria pastinacella* [18] and *Papilio polyxenes* [19]. The safety of synthetic 4-arylcoumarin derivatives still not been tested.

In this study, we examined five synthesized 4-arylcoumarins and twelve 3,4-dihydro-4-arylcoumarins for free radical scavenging activities, antitumor, insect toxicity, hemolysis, and investigated the relationship between their structures and bioactivities, in order to assess their further research potential as drug candidates.

2. Material and methods

2.1. Sources of reagents

The tested 4-arylcoumarins and 3,4-dihydro-4-arylcoumains in our study were synthesized by our research team with the purity substantially ≥98% [6,10]. ABTS and MTT were obtained from Sigma, USA. DPPH was obtained from Woko, Japan. DMSO and butylated hydroxytoluene (BHT) were obtained from Amresco, USA. Sodium nitroprusside (SNP), sulfanilamide, 77 N-(<alpha>-naphthyl)ethylenediamine dihydrochloride (NEDD), Vit C, sodium dodecyl sulfate (SDS), potassium persulfate, and all other reagents were AR purchased from China National Medicines Corporation Ltd., Shanghai, China.

2.2. $ABTS^{\bullet+}$ free radical scavenging activity

Free radical scavenging of compounds was also measured using an improved ABTS radical cation ($ABTS^{\bullet+}$) decolorization assay with modification [20,21]. $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration, both solutions were prepared with distilled water), and incubating the mixture in the dark at 25 °C for 12–16 h before used. For study of the tested compounds, the $ABTS^{\bullet+}$ solution was diluted with absolute ethanol, loaded in 1 cm quartz vessel (BioCell, p/n 7272051, BioTek Instruments Inc., USA), and measured in a micro-plate reader to adjust the absorbance at 734 nm of 0.70 ± 0.02. The diluted $ABTS^{\bullet+}$ solution (180 μL) was mixed with 20 μL of tested compound in 96 well plate. Finally, the plate was quickly loaded into the micro-plate reader at 30 °C, moderately shaken for 5 s, and absorbance at 734 nm measured after 6 min. Ethanol was used as a negative control; Vit C and BHT were used as positive controls. Inhibition percentage was calculated as follows: $100 \times [(A-A_{blank})-(A_c-A_{c\text{-}blank})]/(A-A_{blank})$, where A was the absorbance of only $ABTS^{\bullet+}$ solution, A_{blank} was the absorbance of ethanol, A_c was the absorbance of the examined compound and $A_{c\text{-}blank}$ was the absorbance of ethanol in the presence of compound.

2.3. $NO^{\bullet}$ scavenging capacity

To estimate possible $NO^{\bullet}$ scavenging capacities of the tested compounds, SNP was used as a $NO^{\bullet}$ donor in our experiments. 100 μL SNP (10 mM) was mixed with 100 μL of each compound at set of predetermined concentrations in a 96 well plate. All solutions were prepared with 0.1 M phosphate buffer (PB, pH = 7.4). As SNP is an inorganic complex that can release $NO^{\bullet}$ when exposed to visible light [22,23], the plate was incubated on light (a 5 W 38 lm/W lamp and lighting at the distance of 50 cm in a dark room). After incubation for 120 min at 25 ± 2 °C, Griess' reagent was added to the mixture solution [24]. Briefly, sulfanilamide solution (50 μL, 2% in 5% phosphoric acid) was added, incubated 5 min at room temperature, and then 50 μL NEDD solution (0.1% in distilled water) was added. Finally, the plate was loaded in the micro-plate reader, incubated for 30 min at 25 °C, and the absorbance of wells at 540 nm were measured. For this assay, 100 μL PB was used as a negative control; Vit C and BHT were used as positive controls.

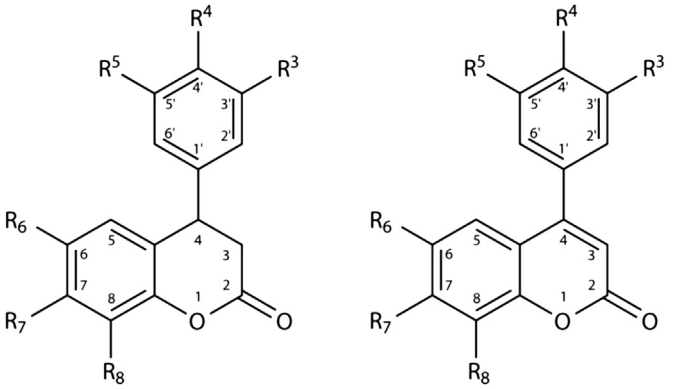
2.4. Antitumor activity

BGC-823 cells (a human stomach cancer cell line) were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 1% antibiotics at 37 °C and 5% CO_2. Cells were trypsinized and resuspended in RPMI 1640 at a concentration of 1×10^5 cells/mL. Cell suspension was seeded in each well of a 96 well plate (50 μL per well) and compounds were dissolved in DMSO solution at a set of predetermined concentrations and diluted 100 times in RPMI 1640. After cells were cultured 2 h, 50 μL compound solutions were added into wells. Mixtures were cultured for 48 h at 37 °C and 5% CO_2. 10 μL MTT dye (5 g/L in PBS) was added into each well and incubated 4 h, then 100 μL SDS (100 g/L in PBS) was added. Absorbance of each well was measured by micro-plate reader at 570 nm [25] after 8 h of culturing. The tumor cell line growth inhibition was calculated as follows: $100 \times (1-A_c/A_n)$, where A_c was the absorbance of test well and A_n was the absorbance of the negative control well. 50 μL cisplatin (10 mg/L) was used as a positive control; 50 μL RPMI1640 (containing 1% DMSO) was used as a negative control. The IC_{50} value was determined as the concentration of compound that caused 50% inhibition of cell proliferation.

2.5. Hemolysis assay

A hemolysis assay [26] was conducted to investigate the cytotoxicity of those coumarins derivatives. Whole rabbit blood was collected by syringe, mixed with EDTA (2 mg/mL) and centrifuged

Table 1
Structures of five 4-arylcoumarins and twelve 4-aryl-3,4-dihydrocoumarins studied in this work.



Compound	R_6	R_7	R_8	R^3	R^4	R^5
4-Arylcoumarins						
1c	H	OH	H	OCH_3	OCH_3	H
1d	H	OH	OH	OCH_3	OCH_3	H
1e	H	OCH_3	H	OCH_3	OCH_3	H
1f	H	OH	OH	OH	OCH_3	H
1g	H	OCH_3	H	H	OCH_3	H
4-Aryl-3,4-dihydrocoumarins						
2a	H	OH	OH	OH	OCH_3	H
2b	H	OH	H	OCH_3	OCH_3	OCH_3
2c	H	OH	H	OCH_3	OCH_3	H
2d	OH	H	H	OCH_3	OCH_3	OCH_3
2e	H	OH	H	OH	OCH_3	H
2f	H	OH	OH	OCH_3	OCH_3	H
2g	H	OH	OH	OCH_3	OCH_3	OCH_3
2h	H	OH	OH	H	OCH_3	H
2i	OH	H	H	H	OCH_3	H
2j	H	OCH_3	H	H	OCH_3	H
2k	H	OCH_3	H	OCH_3	OCH_3	OCH_3
2l	OH	H	H	OCH_3	OCH_3	H

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Table 2
The SC_{50} values of tested compounds.

Compound	SC_{50} (μM)[a]		
	$ABTS^{\cdot+}$	DPPH[b]	$NO^{\cdot}$
1c	8.66 ± 0.7	ND	ND
1d	3.91 ± 0.01	3.24 ± 0.08	133.1 ± 16.5
1e	ND	ND	ND
1f	3.25 ± 0.01	2.63 ± 0.12	63.8 ± 3.2
1g	ND	ND	ND
2a	2.35 ± 0.01	2.69 ± 0.09	105.6 ± 6.4
2b	912.9 ± 64.2	ND	235.7 ± 23.0
2c	162.6 ± 10.6	ND	256.6 ± 7.3
2d	196.3 ± 33.2	925.6 ± 83.3	245.8 ± 49.7
2e	111.8 ± 11.2	247.6 ± 49.3	26.4 ± 0.6
2f	5.03 ± 0.16	3.29 ± 0.12	288.1 ± 27.8
2g	5.48 ± 0.17	3.17 ± 0.12	155.6 ± 27.7
2h	5.48 ± 0.10	2.32 ± 0.10	ND
2i	73.7 ± 14.4	365.2 ± 23.3	ND
2j	ND	ND	ND
2k	ND	ND	ND
2l	95.9 ± 21.2	451.53 ± 64.2	ND
Vit C	9.39 ± 0.20	4.50 ± 0.14	121.9 ± 2.1
BHT	22.6 ± 1.1	143.58 ± 4.62	ND
Resveratrol	0.59 ± 0.04	–	54.6 ± 0.2
Pterostilbene	15.35 ± 1.29	–	ND

ND: not detectable in the tested concentrations.
[a] Confidence limits were 95% and $n = 4$.
[b] The data as reference [6,32].

for 10 min at 700 × *g*. The plasma was removed and blood cells (RBCs) were washed three times with 0.9% saline solution, after which they were suspended in PBS at a concentration of 1×10^8 RBCs/mL. Assayed compounds were dissolved in DMSO solution with set of predetermined concentrations and diluted 50 times with PBS. 100 μL RBCs suspension and 400 μL compounds solution were mixed in 1.5 mL Eppendorf tubes, incubated for 60 min at 37 °C, then centrifuged for 10 min at 12,000 × *g*. The supernatant was collected, and its absorbance was measured at 541 nm. Distilled water was used as the positive control, and PBS (containing 2% DMSO) was used as the negative control. Percent hemolysis, measured at different concentrations of the assayed compounds, was calculated by the following equation: $100 \times (A_c - A_{c\text{-blank}})/(A - A_{blank})$, where A_c was the absorbance of the examined compound, $A_{c\text{-}blank}$ was the absorbance of PBS in the presence of the compound, A was the absorbance of positive control and A_{blank} was the absorbance of PBS.

2.6. Injection and oral toxicity to insect

Injection toxicity of each compound was assayed by the method of Hamamoto et al. [27]. 1 μL compound solutions, dissolved in DMSO and diluted by 20% DMSO, were injected into the hemocoel of *Galleria mellonella* fifth instar larvae (body mass with 250 ± 25 mg) using a micro-injector. An injection of 1 μL 20% DMSO was used as a negative control. Each compound used 20 larvae, and replicated three times for each treatment. Mortality (M%), cocooning (C%), nymphosis (N%) and eclosion (E%) were recorded every 12 h. And the additive effect of insect growth (AG) was calculated as AG(%) = C% + N% + E% − M%. The inhibition effect of compounds to the growth of insects (GI_{int}) was calculated as GI_{int} = (AG of compound − AG of control)/AG of control × 100.

For oral toxicity test, compounds were diluted with absolute ethanol and added into insect diet at maximum concentration of 100 μg/g. The mixed well diets were flattened and dried 30 min to evaporate ethanol, then loaded into 24 well plates (1 g per well). Third-instar larvae were carefully moved into plates (three larvae each well), and were raised in the dark at 30 °C and 65% RH. Larval mortality, weights of surviving larvae, remaining feed, and larvae producing stool were observed and recorded after 10 days. Absolute ethanol was used as negative control. Four replicates of each treatment were made. Relative food consumption rate (RCR), relative growth rate of insects (RGR), approximate digestibility (AD), food digestion and utilization efficiency of conversion of ingested food (ECIF) and other indicators were investigated to assay the compounds toxicity to *G. mellonella*. Nutritional indices were calculated as described previously [28–30] and modified in this study. The following parameters were calculated: RGR = (A−B)/(B × days), where A is the weight of live insects on the tenth day (mg)/number of live insects on the tenth day, B is the original weight of insects (mg)/original number of insects; RCR = *D*/(B × days), where *D* is the biomass ingested (mg)/number of live

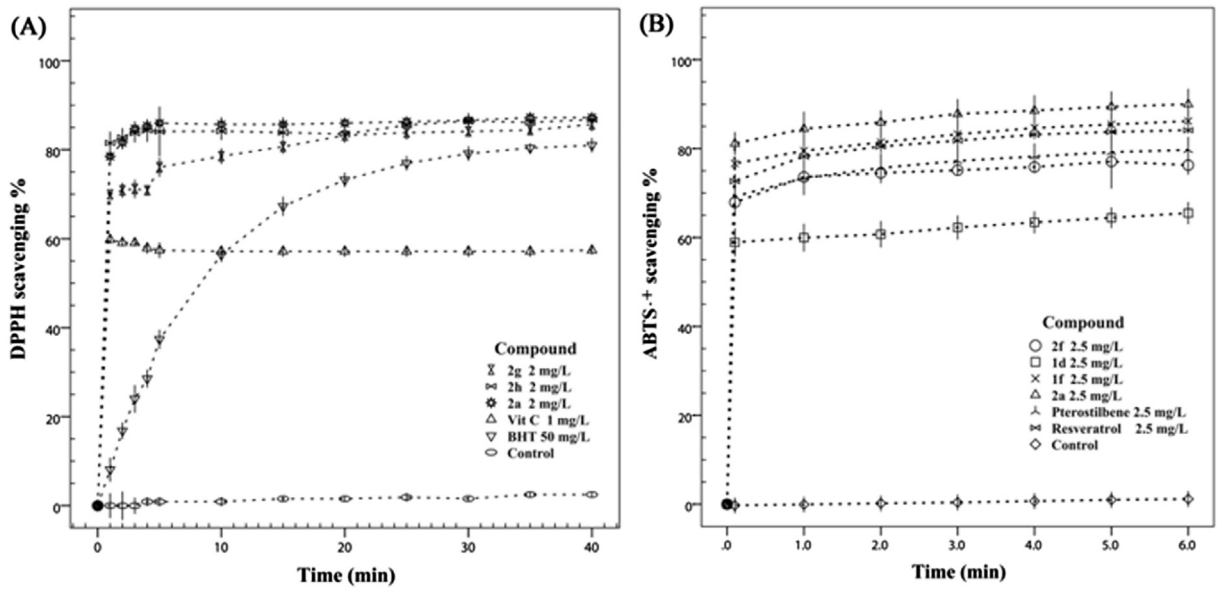

Fig. 1. Kinetic traces of scavenging DPPH (A) and $ABTS^{\cdot+}$ (B) radicals.

insects on the tenth day; AD = dry weight of digested food/dry weight of ingested food × 100; ECIF = (RGR)/(RCR) × 100.

2.7. Lipinski's rule of five

Lipinski's "rule of five" [31] was used to evaluate the drug likeness of those compounds. Generally, orally bioavailable drugs following these rules: number of hydrogen bond donator less than 5; no more than 10 hydrogen bond acceptors; molecular weightless than 500 Da; an octanol–water partition coefficient, log *P* no more than 5.

2.8. Statistical analysis

Statistical analysis was performed with SPSS Statistics software (version 17.0, SPSS Inc. Chicago, USA). One-way ANOVA and Tukey multiple comparison tests were employed to analyze all the results. Results were firsts analyzed with one-way ANOVA, followed by Tukey multiple comparison tests to further examine any significant differences between treatments, at a 0.05 level of significance. All results were expressed as mean ± SD.

Table 3
IC_{50} of compounds in BGC-823 cell line.

Compound	IC_{50}(μM)	Compound	IC_{50}(μM)
1c	120.8 ± 10.1	**2f**	>500
1d	59.0 ± 4.7	**2g**	>500
1e	124.7 ± 16.1	**2h**	237.8 ± 66.4
1f	29.1 ± 1.3	**2i**	444.4 ± 83.9
1g	>500	**2j**	>500
2a	135.8 ± 19.9	**2k**	125.5 ± 16.5
2b	>500	**2l**	>500
2c	>500	Umbelliferone	450.6 ± 61.7
2d	390.9 ± 87.9	Resveratrol	17.1 ± 1.3
2e	214.4 ± 29.6	Pterostilbene	7.3 ± 0.8

3. Results and discussion

3.1. Chemistry of the complexes

Five 4-aryl-3,4-dihydrocoumarin (**1a**–**e**) and twelve 4-arylcoumarins (**2a**–**l**) in this study were synthesized as previously described [6,10,32]. The structures of all the synthesized

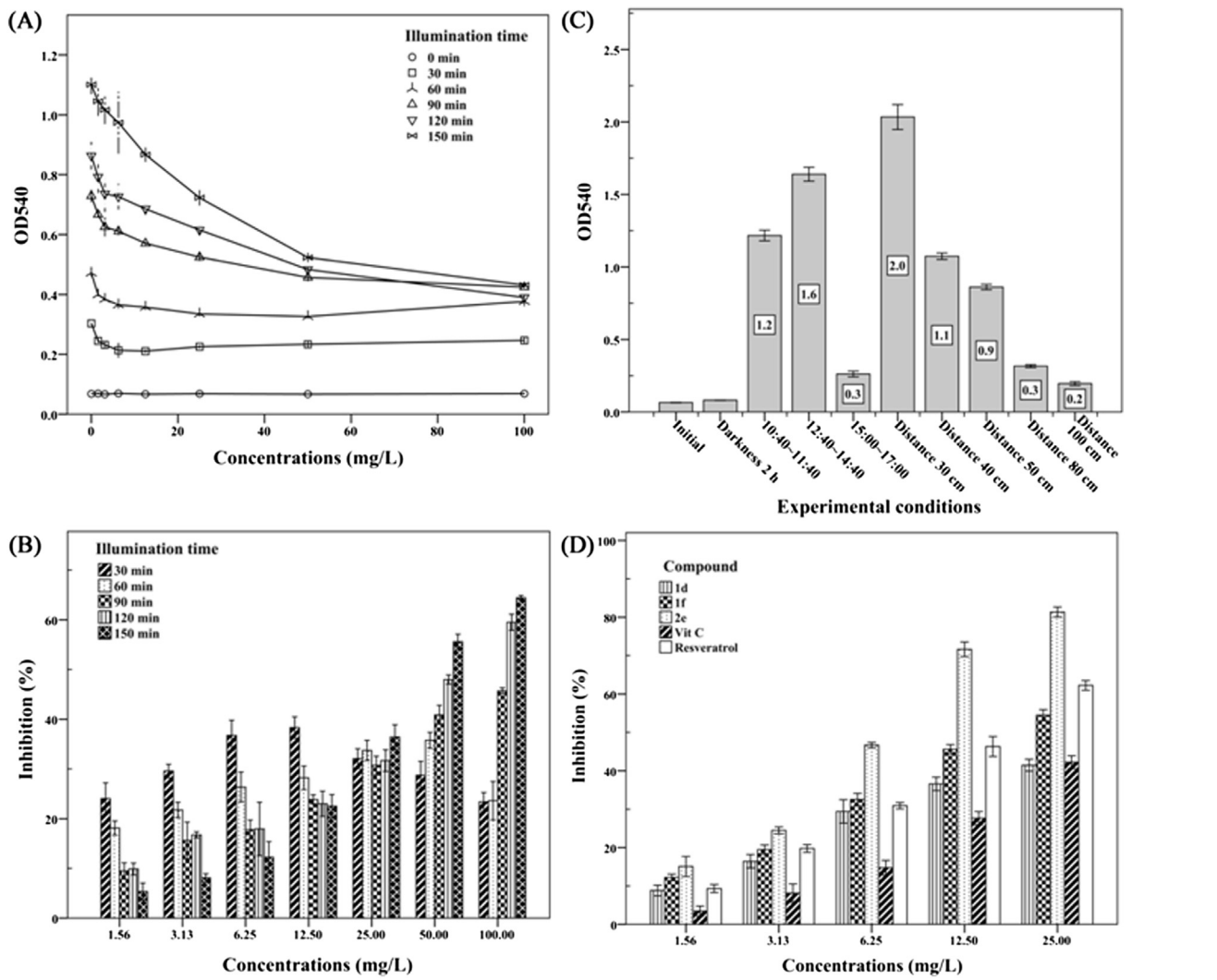

Fig. 2. NO˙ inhibition rate change treated with different concentrations of Vit C at different illumination time (A, B). Distance from light source of 50 cm; light with 5 W 38 lm/W lamp; reaction temperature of 25 ± 2 °C. The NO˙ released were influenced by different illumination conditions (C): in the darkness, radom three time intervals in one day (avoid direct sunlight), serveral distances from the lamp and all the illumination time weresetted120 min. Dose-dependent histograms of several compounds inhibiting the NO˙ generation from SNP (D). The error bars show 95% confidence interval, $n = 4$.

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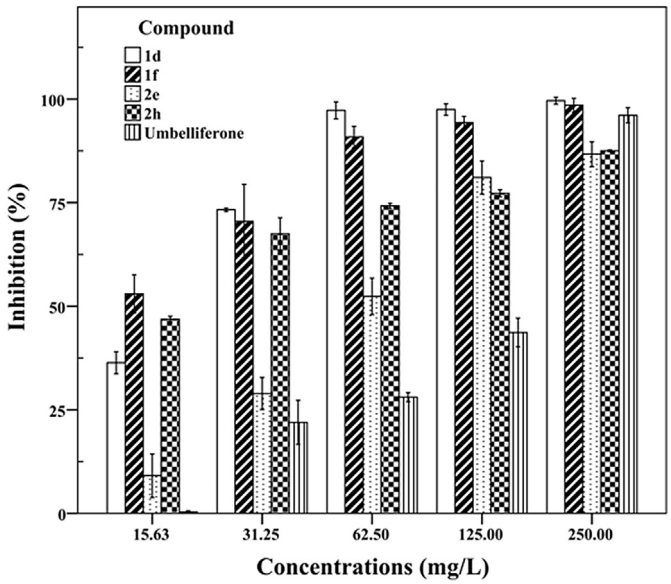

Fig. 3. Dose-dependence histograms of several compounds inhibiting the growth of BGC-823 tumor cells.

compounds were supported by FT-IR, MS, ^{1}H NMR, and ^{13}C NMR spectral data [10,32] (Scheme S1, Tables S1-8).

3.2. *ABTS$^{\bullet+}$ and DPPH free radical scavenging activity*

SC_{50} were calculated to test scavenging ability of those compounds on the two free radicals (Table 2). Results showed that 4-arylcoumarins with 7,8-dihydroxy groups (**1d** and **1f**) or with 7-dihydroxy group (**1c**) and 3,4-dihydro-4-arylcoumarins with 7,8-dihydroxy groups (**2a**, **2f**, **2g** and **2h**) had stronger ABTS$^{\bullet+}$ radical scavenging ability than BHT and Vit C. These results were similar to those for 7,8-dihydroxycoumarins and *ortho* dihydroxycoumarins [5,32,33]. The EC_{50} of compound **2a** was higher than that of **1f** ($P < 0.05$), whereas SC_{50} of compound **1d** was higher than that of **2f**, which suggested that the presence of 3,4-dihydrogen influenced their ABTS$^{\bullet+}$ radical scavenging capacity, which was positively correlated with 3′-hydroxy group (Tables 1 and 2). The SC_{50} value of **2a** was smaller than those of **2f**, **2g** and **2h** ($P < 0.05$), indicating that 3′-hydroxy group in 3,4-dihydro-4-arylcoumarins with 7,8-dihydroxy groups significantly enhanced their ABTS$^{\bullet+}$ scavenging ability, but the number of methoxy groups in those 4-arylcoumarins had no significant correlation with their ABTS$^{\bullet+}$ radical scavenging capacity (Table 2). The SC_{50} values, **2i** < **2l** < **2e** < **2c** < **2d** < **2b**, showed that 3,4-dihydro-4-arylcoumarins with 6-hydroxy group had stronger ABTS$^{\bullet+}$ radical scavenging capacity than those with 7-hydroxy group, and 3,4-dihydro-4-arylcoumarins with more methoxy groups had weaker ABTS$^{\bullet+}$ radical scavenging capacity. The DPPH radical scavenging activity of these compounds were similar to their ABTS$^{\bullet+}$ scavenging activity (Table 2) [6,32]. The DPPH radical scavenging capacities of compounds **2b**, **2c**, and **2e** were similar ($P > 0.05$), which showed that the presence of a 4-(3-hydroxy)-aryl groups in 7,8-dihydroxycoumarins did not significantly enhance their DPPH scavenging ability. The DPPH radical scavenging capacities of **2g** < **2f** < **2h** ($P < 0.05$) and **2d** < **2l** < **2i** ($P < 0.05$) showed that more methoxy groups in 4-aryl group may reduce their antioxidant capability. Furthermore, **2c** > **2l** ($P < 0.01$) and **2b** > **2d** ($P < 0.01$) which indicated a hydroxyl group in the 6th position

Table 4
The percent of RBCs hemolysis after 60 min incubated with different concentrations of the tested compounds at 37 °C.

Compound	100 μg/mL	200 μg/mL	400 μg/mL	700 μg/mL	1000 μg/mL
1c	0.22 ± 0.17	0.98 ± 0.32	7.78 ± 0.29	7.10 ± 0.57	12.48 ± 0.92
1d	ND	ND	0.62 ± 0.37	1.17 ± 0.28	1.98 ± 0.62
1e	2.10 ± 0.76	2.63 ± 0.44	3.58 ± 0.12	4.94 ± 0.58	5.39 ± 0.62
1f	ND	ND	3.18 ± 0.22	5.24 ± 0.76	16.05 ± 1.07
1g	5.73 ± 0.62	11.56 ± 0.18	13.37 ± 0.77	21.71 ± 1.74	43.48 ± 1.36
2a	ND	3.11 ± 0.27	6.98 ± 1.44	12.08 ± 0.36	14.44 ± 0.52
2b	ND	ND	0.54 ± 0.07	3.01 ± 0.27	7.46 ± 0.55
2c	ND	ND	1.02 ± 0.06	10.35 ± 0.83	16.90 ± 1.62
2d	ND	ND	ND	ND	ND
2e	ND	0.43 ± 0.08	1.34 ± 0.17	1.83 ± 0.11	3.74 ± 0.25
2f	1.01 ± 0.04	3.82 ± 0.35	16.23 ± 0.84	26.24 ± 2.34	31.87 ± 0.26
2g	ND	ND	12.03 ± 2.17	25.78 ± 1.69	32.07 ± 2.05
2h	2.01 ± 0. 06	2.56 ± 0.91	17.26 ± 0.98	44.98 ± 2.73	51.42 ± 1.69
2i	ND	ND	8.86 ± 1.75	24.29 ± 3.95	77.02 ± 1.28
2j	6.80 ± 0.29	9.47 ± 0.33	17.66 ± 0.61	21.08 ± 1.16	30.17 ± 0.94
2k	ND	0.61 ± 0.42	2.01 ± 0.04	8.44 ± 0.39	12.72 ± 0.61
2l	0.38 ± 0.06	1.06 ± 0.18	1.98 ± 0.09	3.32 ± 0.37	6.12 ± 0.74
Umbelliferone	ND	ND	ND	1.58 ± 0.21	7.81 ± 0.33
Resveratrol	ND	ND	10.24 ± 0.26	15.53 ± 0.31	24.27 ± 0.17
Pterostilbene	100	100	100	100	100

ND : no detectable hemolysis in the tested concentrations.

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Table 5
The inhibition effect of compounds to the growth state of *G. mellonella* after injection.

Compound	24 h		96 h		120 h		240 h	
	AG	GI_{int}	AG	GI_{int}	AG	GI_{int}	AG	GI_{int}
1c	100	0	160	11	180	10	230	0.8
1d	90	10	130	28	140	30	220	1.7
1e	90	10	140	22	170	15	210	16
1f	90	10	170	12	200	0	250	0
1g	90	10	150	17	200	0	240	0.4
2a	60	40	120	33	180	10	230	0.8
2b	90	10	180	0	200	0	250	0
2c	100	0	180	0	200	0	250	0
2d	90	10	180	0	200	0	250	0
2e	90	10	140	22	180	10	230	0.8
2f	90	10	140	22	190	5	240	0.4
2g	90	10	150	17	180	10	230	0.8
2h	100	0	160	11	200	0	250	0
2i	90	10	120	33	160	20	200	20
2j	80	20	130	28	180	10	230	0.8
2k	60	40	110	39	160	20	210	16
2l	90	10	180	0	190	5	240	0.4
Umbelliferone	60	40	100	44	120	40	140	44
Resveratrol	90	10	180	0	200	0	250	0
Pterostilbene	60	40	130	28	140	30	200	20

conferred stronger radical scavenging ability than in the 7th position (Table 2). The kinetic curves of DPPH and $ABTS^{\bullet+}$ radical scavenging indicated that the scavenging process was very fast and plateaued in 1 min (Fig. 1).

3.3. $NO^{\bullet}$ scavenging capacity

As the generation of $NO^{\bullet}$ from SNP is a photolytic reaction [34], illumination time significantly influences the result of an $NO^{\bullet}$ radical scavenging capacity experiment. We firstly used Vit C to determine an optimal illumination time of 2 h (Fig. 2). The $NO^{\bullet}$ radical scavenging capacity assay showed that 4-arylcoumarins with 7,8,3′-trihydroxy-4′-methoxy groups (**1f**) and 3,4-dihydro-4-arylcoumarin with 7,8,3′-trihydroxy-4′-methoxy groups (**2a**) had stronger $NO^{\bullet}$ radical scavenging activities than Vit C. **1f** had almost double the $NO^{\bullet}$ radical scavenging capacity of **2a**, which indicated that the presence of a 3,4-dihydro groups significantly reduced the $NO^{\bullet}$ radical scavenging capacity of 4-arylcoumarins. 3,4-Dihydro-4-arylcoumarin with 7,3′-dihydroxy and 4′-methoxy groups (**2e**) had the strongest $NO^{\bullet}$ radical scavenging capacity. The SC_{50} values, **2e** < **2a** ($P < 0.05$), **2c** < **2f** ($P < 0.05$) and **2b** < **2g** ($P < 0.05$) suggested that the presence of a 7-hydroxy group was essential for the $NO^{\bullet}$ radical scavenging capacity of 3,4-dihydro-4-arylcoumarins, whereas an 8-hydroxy group reduced the scavenging effectiveness of those compounds. The series of SC_{50} values, **2g** < **2b** < **2d** < **2c** < **2f** ($P < 0.05$) indicated that 3,4-dihydro-4-arylcoumarins with trimethoxy groups had stronger $NO^{\bullet}$ radical scavenging capacity than those with dimethoxy groups.

3.4. Antitumor activity

The MTT assay was used to assess antitumor activity in 4-arylcoumarins and 3,4-dihydro-4-arylcoumarins (Table 3). The series of IC_{50}values, **1f** < **1d** < **1c** < **1e** < **2k** < **2a** ($P < 0.05$) showed that without 3,4—dihydrogen groups and more hydroxyl groups in 4-arylcoumarins enhanced their antitumor activity. Of the seventeen 4-arylcoumarins, compound **1f** had the strongest antitumor activity with an IC_{50} of 29.1 ± 1.3 μM inhibited the proliferation of BGC-823 cells, while a concentration of 62.5 mg/L was almost completely lethal to all cells (Fig. 3).

3.5. Hemolysis

Red blood cells (RBCs) of rabbit were used as target to investigate the cytotoxicity of our 4-arylcoumarin compounds. None of the seventeen compounds showed strong cytotoxicity toward RBCs (Table 4). Of all the compounds, compound **2j** had the highest cytotoxicity and only induced 6.8% RBCs hemolysis at 100 μg/mL. Compounds **2a**, **2f** and **2e** (200 μg/mL) had very weak cytotoxicity to RBCs and induced less than 3.82% RBCs hemolysis. Compounds **1d**, **1f**, **2b**, **2c**, **2d**, **2g** and **2i** showed no detectable cytotoxicity to RBCs even with concentration 200 μg/mL.

3.6. Injected and oral toxicity to insect

G. mellonella larvae were used as targets to estimate injection and oral toxicity of 4-arylcoumarins and 3,4-dihydro-4-arylcoumarins to insect. Results showed that none of the tested

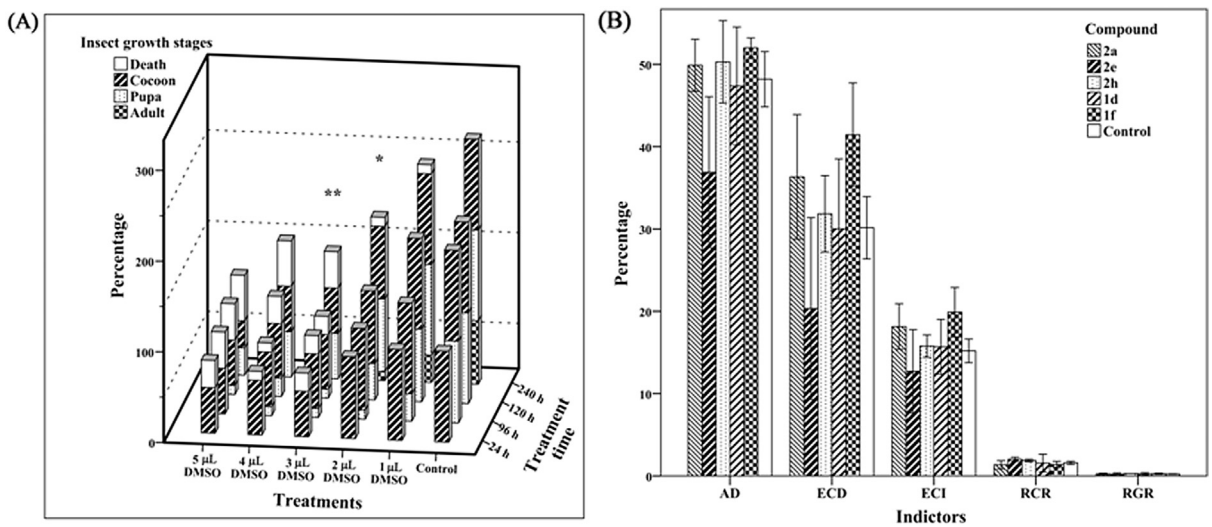

Fig. 4. Influence of DMSO on growth and development of *Galleria mellonella* (A) and oral toxicity of two 4-arylcoumarins and three 3,4-dihydro-4-arylcoumarins against *G. mellonella* (B). $^{*}P < 0.05$, $^{**}P < 0.001$ compared with control.

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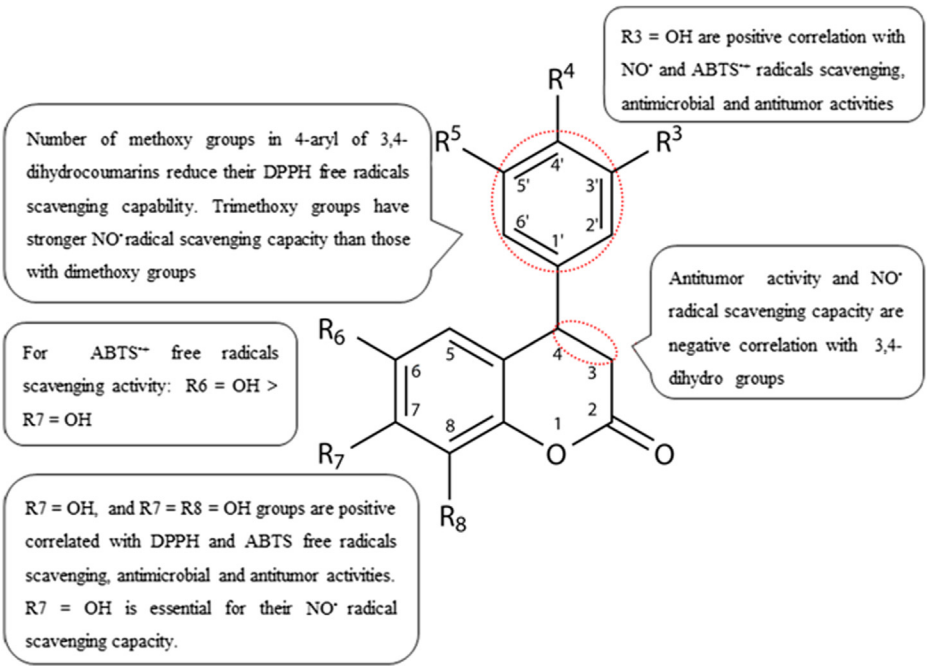

Fig. 5. The structure–activity relationships in tested 4-arylcoumarins and 3,4-dihydro-4-arylcoumarins.

compounds significantly affected larval viability. No significant mortality and no appetite-suppressant effect were detected in the tested larvae (Table 5, Fig. 4).

3.7. Structure–activity relationships of seventeen novel 4-arylcoumarin derivatives

For free radical scavenging activity, 4-arylcoumarin derivatives containing 7,8-dihydroxy groups, 6-hydroxy group, 7-hydroxy group and 3′-hydroxy group were positively correlated with $ABTS^{\bullet+}$ and DPPH free radical scavenging activities [6] (Fig. 5). Compounds containing 7-hydroxy and 3′-hydroxy groups were positively correlated with NO· free radical scavenging activity, whereas the presence of an 8-hydroxy group was negatively correlated (Fig. 5).

4-Arylcoumarin compounds containing 7,8-dihydroxy and 3′-hydroxy groups were positively correlated with antitumor activity. However, compounds containing 3,4-dihydro groups showed significantly reduced antitumor activity (Fig. 5).

Compounds containing 3′-hydroxy, 6-hydroxyl, 7,8-dihydroxy and 7-methoxy groups together with 3,4-dihydro groups were correlated with increased antimicrobial activities [10] (Fig. 5). These compounds with only 7-hydroxy group showed no detectable antimicrobial activities. Compounds with 3,4-dihydro groups had stronger antimicrobial activity against gram-negative bacteria and weaker antimicrobial activities against gram-positive bacteria and fungi than unmodified 4-arylcoumarin compounds [10] (Fig. 5).

Table 6
Some common molecular descriptors of tested compounds.

Compound[a]	MW	AlogP	H-bond Acceptors	H-bond donors
1c	298.29	3.103	5	1
1d	314.289	2.861	6	2
1e	312.317	3.329	5	0
1f	300.263	2.635	6	3
1g	282.291	3.345	4	0
2a	302.279	2.464	6	3
2b	330.332	2.915	6	1
2c	300.306	2.932	5	1
2d	330.332	2.915	6	1
2e	286.279	2.706	5	2
2f	316.305	2.69	6	2
2g	346.331	2.673	7	2
2h	286.279	2.706	5	2
2i	270.28	2.948	4	1
2j	284.307	3.174	4	0
2k	344.358	3.141	6	0
2l	300.306	2.932	5	1

[a] all the compounds absolutely obeyed the “rule of five”.

3.8. Potential of 4-arylcoumarin derivatives as novel drug candidates

Two 4-arylcoumarins, **1f** and **1d**, and four 3,4-dihydro-4-arylcoumarins, **2a**, **2f**, **2g** and **2h**, had stronger $ABTS^{\bullet+}$ and DPPH free radical scavenging activities than those of Vit C and pterostilbene. Kinetic traces of radical scavenging showed that scavenging of DPPH radicals by compounds **2a**, **2g** and **2h** were fast, occurring 1 min into the platform stage. This was also the case for scavenging of $ABTS^{\bullet+}$ radicals by compounds **2a**, **2f**, **1d** and **1f** (Fig. 3). 7,3′-Dihydroxy-4′-methoxy-3,4-dihydro-4-arylcoumarin, **2e**, had stronger NO· radical scavenging activity than that of Vit C, resveratrol and pterostilbene, but with much weaker hemolytic activity against vertebrate blood cells than that of the latter three compounds. 7,8,3′- Trihydroxy-4′-methoxy-4-arylcoumarin, **1f**, showed similar antitumor activity as resveratrol and very weaker hemolysis activity to vertebrate blood cells than that of resveratrol and pterostilbene. Four 3,4-dihydro-4-arylcoumarin derivatives, **2a**, **2f**, **2g** and **2h**, had significant antimicrobial activity against gram-negative bacteria, with similar minimal inhibitory concentration as ampicillin, and a three times weaker inhibition effect against probiotics (such as *Bacillus subtilis*) than that of ampicillin [10]. All of these compounds may have well drug-likeness potential for none of them violated the rule of five (Table 6).

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4. Conclusion

In conclusion, two novel 4-arylcoumarins, **1f**, **1d**, and five novel 3,4-dihydro-4-arylcoumarins, **2a**, **2f**, **2g**, **2h** and **2e** showed potent in vitro bioactivity (free radical scavenging, antitumor activity against BGC-823 cells, antibacterial effects) and were not toxic against vertebrate blood cell or insect. Our results indicated that these compounds may are potential therapeutic candidates for pathological conditions characterized by free radical overproduction.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.03.014.

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