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ARTICLE

# Synthesis, and antioxidant, thrombin-inhibitory, and anticancer activities of hydroxybenzamide derivatives

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**Abstract** Four hydroxybenzamide derivatives were synthesized from protocatechuic acid. The antioxidant activities of these derivatives were evaluated in vitro using DPPH radical scavenging and reducing power assays. In addition, these compounds were subjected to a chromogenic thrombin-inhibitory assay using Chromozym-TH as the substrate. The anticancer effects of these derivatives were also investigated using the MTT assay in three human cancer cell lines. Four derivatives exhibited excellent DPPH radical scavenging activity and redox potential. Their highly inhibitory effect against thrombin may allow them to be utilized as novel micromolecule thrombin inhibitors. Furthermore, four derivatives demonstrated different inhibitory activities in three human cancer cell lines but showed minimal harmful effects on normal HUVEC cells. Therefore, hydroxybenzamide derivatives indicate the superior antioxidant, thrombin-inhibitory, and anticancer activities along with the excellent biocompatibility, which have the potential application in therapeutic interventions and disease prevention of cancer, thrombotic diseases, and aging.

**Keywords** Antioxidant activity · Anticancer activity · Hydroxybenzamide derivatives · Protocatechuic acid · Thrombin inhibitory

## Introduction

Cancer and thrombotic diseases are the highest mortality rate of disease in the past few decades, which have attracted the large amounts of funds and research to conquer these problems (Ilies et al. 2002; Okuyama et al. 1997). There are a spectrum of therapeutic methodologies for the treatment of cancer, including surgery, chemotherapy, radiotherapy, hyperthermia, and photodynamic therapy (Liang et al. 2015). However, the above methodologies are rarely sufficient to overcome cancers for the limit of each individual therapeutic route, which is also the predicament for the treatment of thrombotic diseases (Liang et al. 2014). Moreover, following the increase of environmental pollution and social pressure, reactive oxygen species (ROS) in the body will enhance obviously, which results in the senescence and is also the inducement of various diseases such as the cancer (Stich and Anders 1989) and thrombotic diseases (Iuliano et al. 1994). Recently, increasing attention has focused on the preventive medicine and health products from foods or some herbaceous plants, which would effectively prevent the above diseases (Chang and So 2008). The focus has been gradually shifted away from the basic supplements of nutrition and energy to health-enhancing ingredients with

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multifaceted properties (Masella et al. 2012). Thus, how to extract and synthesize the bioactive and avirulent constituents from plants and foods will tremendously promote development of prevention of cancer and thrombotic diseases.

In many herbal and traditional medicines, phenolic acids are often treated as the bioactive constituents, which as the secondary plant metabolites are universally present in nature, plants, and plant-derived foods (Harborne 1980). In our previous research, a series of phenolic acids and their derivatives bound to bovine serum albumin with high avidity, indicate that they might be carried by serum albumin to therapeutic targets (Luo et al. 2013; Meng et al. 2012; Wei et al. 2014). Protocatechuic acid (3,4-dihydroxybenzoic acid) contains hydroxybenzoic structures in naturally occurring phenolic acids and is present in many plants (Shahidi et al. 1992). Protocatechuic acid possesses a wide spectrum of biological effects, including antioxidative (Kayano et al. 2002), free-radical quenching (Tseng et al. 1996), and antimicrobial properties (Alves et al. 2013). Therefore, it can inhibit oxidation processes and protect the body from damaging oxidation reactions, which may have beneficial effects in health promoting, inflammation (Wang et al. 2014), neuroprotective influences (Guan et al. 2006), and mutagenesis leading to carcinogenesis (Tanaka et al. 2011; Tseng et al. 1998; Yin et al. 2009). Moreover, protocatechuic acid would enhance the antiplatelet effect in therapeutic interventions due to the fact that reactive oxygen species play a crucial role in blood coagulation (Iuliano et al. 1997; Pignatelli et al. 1998). Furthermore, protocatechuic acid is an apoptosis inducer in human leukemia cells (Tseng et al. 2000) and possesses antiatherogenic (Wang et al. 2012) and cardioprotective effects (Semaming et al. 2014). In addition, thrombin plays a pivotal role in the processes of hemostasis and thrombosis (Hemker 1993). A safe and effective inhibitor of thrombin might be utilized in treatments for venous thrombosis, atrial fibrillation, restenosis, arterial thrombosis, and for the prevention of myocardial infarction (Kimball 1995). A previous study showed that protocatechuic acid reduced plasminogen activator inhibitor-1 activity and fibrinogen levels (Lin et al. 2009), demonstrating a superior anticoagulatory effect, which indicated that protocatechuic acid would possess the ability of inhibition of thrombin activity. The above discussion indicated that protocatechuic acid contained multifunctional activities such as antioxidation, free-radical scavenging, anticancer, and inhibition of thrombin activity. Therefore, how to synthesize the more effectiveness of protocatechuic acid derivatives with the improved activities will promote the development of medical science and preventive medicine.

In this study, the amide derivatives of 3,4-dihydroxybenzoic acid (**1**) were synthesized, while compounds **WF**, **WN** were first obtained. The amido providers were *p*-aminomethylbenzoic acid, tranexamic acid, 6-aminocaproic acid, and taurine, which all have the amido and the carboxyl active group (sulfonic group) in the structure. The differing characteristics of the amido providers were the length of the carbon chain and the unsaturated or saturated hexatomic ring between the aminomethylene group and the terminal carboxylic (sulfonic group). The multifunctional activities of the hydroxybenzamide structure prompted us to conduct a study to evaluate some of the activities exerted by these derivatives. The antioxidant activities of these derivatives were examined using DPPH radical scavenging and reducing power assays, which indicated that compounds **1** and its derivatives possessed the superior antioxidant activities. A novel evaluation of the inhibitory effects of protocatechuic acid and its amide derivatives on thrombin was performed using the chromogenic method (Chromozym-TH), and was found that compounds **1** and hydroxybenzamide derivatives **WH**, **WN** exhibited thrombin inhibition ability. Moreover, in vitro antitumor activities of these derivatives against three tumor cell lines, A2780 (human ovarian carcinoma cells), MCF-7 (human breast cancer cells), K562 (human chronic myelogenous leukemia cells), were evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Compounds **1** and four derivatives have the well anticancer activity and excellent biocompatibility, which have the potential application in the prevention and treatment of cancer. Therefore, in view of their wide range of pharmacological and biological activities, these novel hydroxybenzamide derivatives have great potential in food supplements or health product for therapeutic interventions and disease prevention of cancer, thrombotic diseases, and aging.

## Materials and methods

### Materials

DPPH (2,2-diphenyl-1-picrylhydrazyl) was biological reagent, purchased from Aladdin-reagent (Shanghai, China). Thrombin (≥1000 NIH units/mg protein) prepared from human plasma is purchased from Sigma-Aldrich (USA). Chromozym-TH is biological and purchased from Roche (Swiss). Hirudin was obtained from Lianhe Botai biological technology company (Dalian, China). All other reagents were commercial materials of analytical purity without further purification.

$^{1}H$ and $^{13}C$ NMR spectra were carried out using a Bruker Avance III HD 600 NMR spectrometer in DMSO-$d_6$ or $D_2O$ solution. Infrared spectra were performed as KBr sheets on a Tensor 27 FTIR spectrometer (Bruker,

Germany). ESI–MS were collected using a LC–MS 2010A (Shimadzu, Japan). The melting points were measured on an X-4 microscopic melting-point apparatus (Beijing Technology Instruments Company, China) and were uncorrected. The UV absorption spectra were recorded on a 7600 double-beam UV/visible spectrophotometer (Shanghai Jinghua Instruments, China) using a 1.0-cm quartz cell. Enzyme inhibition reacted with drugs was monitored using a BIO−RAD iMark microplate reader.

## Synthesis procedures

3,4-Dihydroxybenzoic acid (**1**, 50.0 g) was dissolved in acetic anhydride (250 mL). After adding 10 mL of pyridine, the reaction mixture was stirred at 120 °C for 5 h. The excess solvent was evaporated off. Water was added, and then the crude intermediate 3,4-diacetoxybenzoic acid (**2**) was precipitated out.

Compound **2** (7.6 g, 0.032 mol) was dissolved in 10 mL of thionyl chloride. While stirring, four drops of dimethylformamide were added, and then the mixture was refluxed for 5 min at 70 °C. The excess thionyl chloride was removed, and the liquid residue (**3**) was dispersed in 10 mL of THF (Scheme 1).

The obtained solution of compound **3** was added dropwise to a water (30 mL) solution of 0.016 mol amino provider (*p*-aminomethylbenzoic acid 2.4 g, tranexamic acid 2.5 g, 6-aminocaproic acid 2.1 g, or taurine 2.0 g), sodium carbonate (0.9 g, 0.008 mol), and sodium bicarbonate (1.3 g, 0.016 mol) at 10–15 °C. The pH value was kept between 7 and 8 by the addition of sodium bicarbonate. The mixture was stirred at room temperature until TLC indicated that the reaction was completed by ninhydrin coloring. The mixture was acidified with hydrochloric acid to pH 4–5, and then the solvents were evaporated off. The residue was washed three times with ethyl acetate. Purification of the crude product by silica gel column chromatography or by recrystallization in water yielded four hydroxybenzamide derivatives. The synthetic routes to hydroxybenzamide derivatives are outlined in Scheme 2.

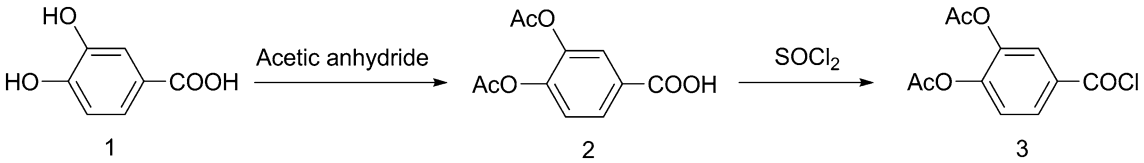
## Data for hydroxybenzamide derivatives

4-((3,4-Dihydroxybenzamido)methyl)benzoic Acid (**WF**). Dark yellow powder, yield: 32.05 %, m.p. >300 °C. $^{1}$H-NMR (DMSO-$d_6$, 600 MHz) $\delta$: 8.78 (t, $J$ = 5.9 Hz, 1H, NH), 7.89 (d, $J$ = 8.1 Hz, 2H, H-2′,6′), 7.35 (d, $J$ = 2.1 Hz, 1H, H-2), 7.34 (d, $J$ = 8.1 Hz, 2H, H-3′,5′), 7.25 (dd, $J$ = 2.1, 8.3 Hz, 1H, H-6), 6.78 (d, $J$ = 8.3 Hz, 1H, H-5), 4.71 (2H, 3-OH, 4-OH), 4.47 (d, $J$ = 5.9 Hz, 2H, $CH_2$). $^{13}$C-NMR (DMSO-$d_6$, 150 MHz), $\delta$: 175.2 (COOH), 166.4 (CONH), 148.9 (C-4), 145.2 (C-3), 144.1 (C-4′), 132.0 (C-1), 129.4 (C-2′,6′), 126.9 (C-3′,5′), 125.4 (C-1′), 119.2 (C-6), 115.5 (C-5), 115.3 (C-2), 42.5 ($CH_2$). IR (KBr) ν: 3424 (Ph-OH), 1630 (amide C=O), 1552 (carboxyl C=O) cm$^{-1}$. MS (ESI) *m/z*: 286.2 (M–1)$^-$.

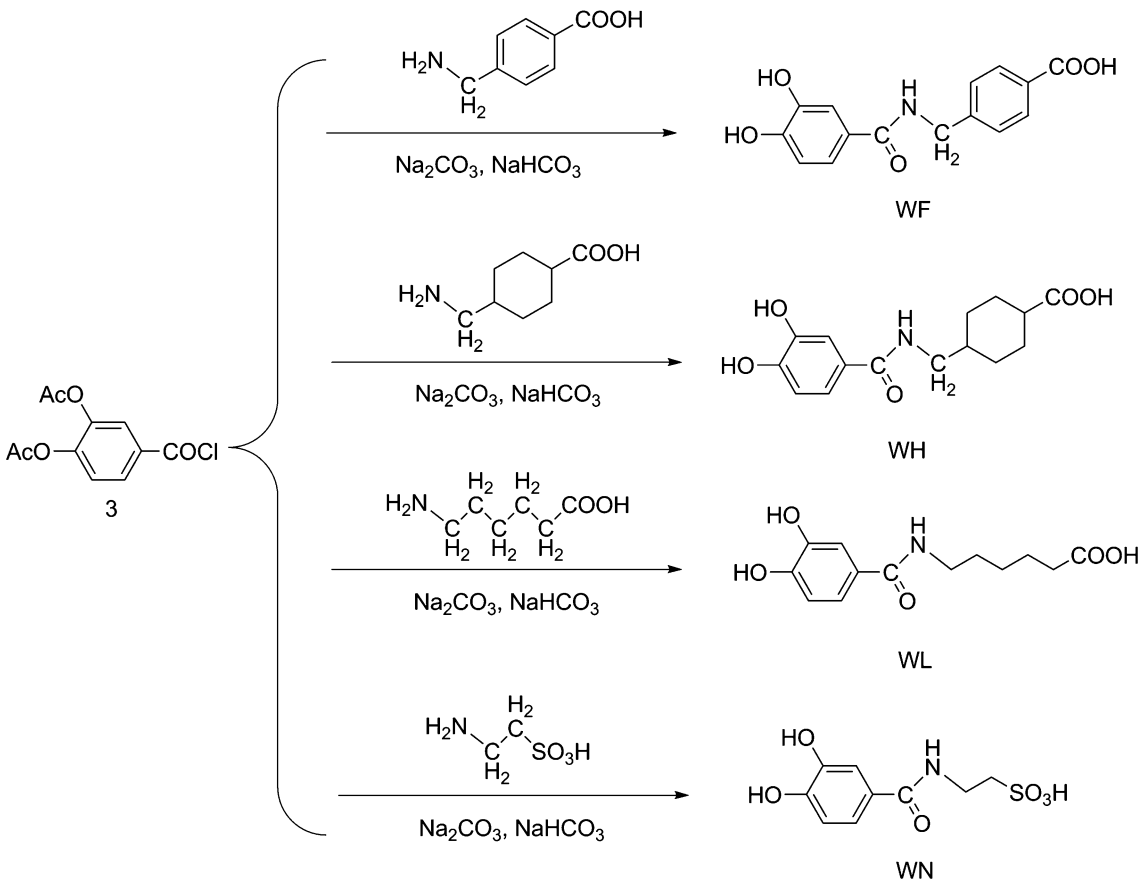
4-((3,4-Dihydroxybenzamido)methyl)cyclohexane-1-carboxylic Acid (**WH**). Dark yellow powder, yield: 13.58 %, m.p. 98–100 °C. $^{1}$H-NMR (DMSO-$d_6$, 600 MHz) $\delta$: 8.08 (t, $J$ = 5.2 Hz, 1H, NH), 7.25 (d, $J$ = 1.0 Hz, 1H, H-2), 7.14 (m, 1H, H-6), 6.71 (d, $J$ = 8.2 Hz, 1H, H-5), 3.02 (t, $J$ = 5.2 Hz, 2H, $CH_2$), 1.97 (m, 1H, H-1′), 1.85, 1.21 (dd, $J$ = 23.3, 11.6 Hz, 4H, H-2′,6′), 1.44 (m, 1H, H-4′), 1.72, 0.88 (dd, $J$ = 11.6, 11.5 Hz, 4H, H-3′,5′). $^{13}$C-NMR (DMSO-$d_6$, 150 MHz), $\delta$: 174.8 (COOH), 166.3 (CONH), 149.4 (C-4), 145.7 (C-3), 125.4 (C-1), 118.9 (C-6), 115.7 (C-5), 115.6 (C-2), 45.3 ($CH_2$), 44.6 (C-1′), 37.4 (C-4′), 30.2 (C-2′,6′), 29.2 (C-3′,5′). IR (KBr) ν: 3397 (Ph-OH), 1629 (amide C=O), 1560 (carboxyl C=O) cm$^{-1}$. MS (ESI) *m/z*: 292.3 (M–1)$^-$.

6-(3,4-Dihydroxybenzamido)hexanoic Acid (**WL**). Dark yellow powder, yield: 12.36 %, m.p. 198–200 °C. $^{1}$H-NMR (DMSO-$d_6$, 600 MHz) $\delta$: 8.10 (t, $J$ = 5.1 Hz, 1H, NH), 7.25 (d, $J$ = 1.6 Hz, 1H, H-2), 7.13 (dd, $J$ = 8.2, 1.6 Hz, 1H, H-6), 6.71 (d, $J$ = 8.2 Hz, 1H, H-5), 3.16 (td, $J$ = 6.6, 5.1 Hz, 2H, H-6′), 2.03 (t, $J$ = 7.4 Hz, 2H, H-2′), 1.47 (m, 4H, H-3′,5′), 1.25 (m, 2H, H-4′). $^{13}$C-NMR (DMSO-$d_6$, 150 MHz), $\delta$: 175.3 (C-1′), 166.3 (CONH), 149.4 (C-4), 145.4 (C-3), 125.3 (C-1), 118.7 (C-6), 115.6 (C-2), 115.5 (C-5), 36.8 (C-6′), 29.2 (C-2′), 26.7 (C-5′), 25.6 (C-4′), 24.0 (C-3′). IR (KBr) ν: 3373 (Ph-OH), 1631 (amide C=O), 1556 (carboxyl C=O) cm$^{-1}$. MS (ESI) *m/z*: 266.3 (M–1)$^-$.

2-(3,4-Dihydroxybenzamido)ethane-1-sulfonic Acid (**WN**). Dark yellow powder, yield: 24.74 %, m.p. >300 °C. $^{1}$H-NMR ($D_2O$, 600 MHz) $\delta$: 7.23 (s, 1H, H-2), 7.19 (m, 1H, H-6), 6.91 (m, 1H, H-5), 3.73 (m, 2H, H-2′), 3.19 (m, 2H, H-1′). $^{13}$C-NMR ($D_2O$, 150 MHz), $\delta$: 169.7 (CONH), 147.9 (C-4), 143.8 (C-3), 125.6 (C-1), 120.4 (C-6), 115.7 (C-5), 114.9 (C-2) 49.7 (C-1′), 35.6 (C-2′). IR (KBr) ν: 3467 (NH), 1640 (amide




**Scheme 1** Synthetic routes to 3,4-dihydroxybenzoic acid intermediate **3**




**Scheme 2** Synthetic routes to hydroxybenzamide derivatives **WF**, **WH**, **WL**, and **WN**

C=O), 1255, 1173, 1067 ($SO_3H$) $cm^{-1}$. MS (ESI) *m/z*: 260.1 $(M–1)^-$.

## Scavenging activity of DPPH radical

The DPPH radical scavenging assay proposed by Blois (1958) was employed to measure the antioxidant activity of the obtained compounds. A series of different volumes of hydroxybenzamide derivative solutions (1 mM, in ethanol), varying from 0 to 1.0 mL, was mixed with 1.0 mL of DPPH solution (1 mM, in ethanol) in colorimetric tubes and then diluted to 10 mL with ethanol. After 30 min away from light, the absorbance $A_i$ was determined at 517 nm. In the same manner, the different volumes of test compound solutions were diluted to 10 mL in ethanol without DPPH, and the absorbance $A_j$ was collected. Ascorbic acid was used as a positive control. All the determinations were performed in triplicate. The DPPH radical scavenging activities (RSA, %) of the test compounds were calculated using the following equation:

$$\text{RSA}\,(\%) = 1 - \frac{A_i - A_j}{A_0}, \quad (1)$$

where $A_i$ is the absorbance after a 30-min incubation of the test compounds with DPPH, $A_j$ is the absorbance of the test compound solutions (with final concentrations ranging from 0.05 to 0.1 mM) without DPPH, and the blank control $A_0$ is the absorbance of the DPPH solution without the test compounds.

Kinetics assays of the scavenging effect on the DPPH radical by compounds **1** and four derivatives were also performed using the same method described above. Seven different volumes (100, 200, 300, 400, 500, 800, and 1000 μL) of test compound solutions (1 mM) were used. The absorption at 517 nm was measured immediately after shaking and was read at 5-min intervals for 40 min. Kinetics curves were then recorded according to the radical scavenging activity time.

## Reducing power determination

Oyaizu (1986) introduced a reducing power measurement. In this assay, the determination of reducing power was based on Hayat's method (Hayat et al. 2010). A series of 1.0 mL volumes of test compound solutions at different

concentrations (0–1.0 mM, in water) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1 % w/v). The mixtures were incubated at 50 °C for 20 min, and then 2.5 mL of trichloroacetic acid (10 % w/v) was added. After 10 min of centrifugation, 2.5 mL of the supernatant was added into tubes with 2.5 mL of distilled water and 0.5 mL ferric chloride (0.1 % w/v). The absorbance of the final mixture was recorded at 700 nm. Ascorbic acid was used as a positive control. The blank control contained all the reagents except the test compounds. All measurements were performed in triplicate.

### Determination of the thrombin-inhibitory activity

The thrombin inhibition assay was conducted using the chromogenic substrate method (Chang 1983). The substrate Tos-Gly-Pro-Arg-pNA (Chromozym-TH) (Lottenberg et al. 1982) can be hydrolyzed by thrombin and cleaved into two parts, a residual peptide and a yellow substance, *p*-nitroaniline, which can be measured in a microplate spectrophotometer. Twenty microliters of thrombin solution (1 U/mL, in 0.1 % bovine serum albumin, pH 6.5) was mixed with 20 μL of test compound solution (dissolved in 50 mM Tris buffer, pH 8.3) at different concentrations (0.625, 1.25, 2.5, 5, and 10 mg/mL). The mixture was incubated at 37 °C for 10 min before the addition of 20 μL of Chromozym-TH solution (2 mM). After another 10 min incubation at 37 °C, 50 μL of acetic acid (50 % w/v) and 40 μL of water were successively added to terminate the reaction. For each concentration, three replicate wells were used. The absorbances were monitored at 415 nm. Hirudin was used as a positive control. The Tris-treated controls were considered to have a thrombin activity value of 100 %. The $IC_{50}$ values represent the concentrations that caused 50 % inhibition relative to the Tris-treated controls.

### Cell culture and in vitro growth-inhibition assay

Human ovarian carcinoma A2780 cells, human breast cancer MCF-7 cells, and human chronic myelogenous leukemia K562 cells were obtained from the Guangxi Cancer Research Institute and cultured in RPMI 1640 medium (GIBCO) supplemented with 10 % fetal bovine serum (GIBCO), 100 U/mL penicillin (Solarbio), and 100 U/mL streptomycin (Solarbio) at 37 °C with 5 % $CO_2$. Cell cultures were passaged every 2–3 days using trypsin (Solarbio) to detach the cells from their culture flasks. The cancer drug doxorubicin was used as a positive control. All test compounds were dissolved in triple-distilled water at 10 mM immediately before use and were diluted in medium before addition to the cells.

The three different cell types were used to evaluate the inhibitory effect of compounds **1** and four derivatives on cell growth using an MTT assay adapted from the literature (Alley et al. 1988; Mosmann 1983). Cells were seeded on 96-well microplates in 100 μL of medium ($1 \times 10^5$ cells/mL) and were incubated for 12–24 h to allow cell attachment. Next, the culture medium supernatant was removed from the wells, and the cells were treated with 200 μL of test compound (0.31, 0.62, 1.25, 2.5, and 5 mM) for 48 h. For each concentration, three replicate wells were used. After the culture medium supernatant was again removed, the cells were treated with 20 μL of MTT solution (5 mg/mL) and 180 μL of complete medium and were then incubated at 37 °C for 4 h. The medium was aspirated, and dimethyl sulfoxide (100 μL) was added to each well to dissolve the formazan solution. The absorbances of the plates were read using a test wavelength of 490 nm (with 620 nm as a reference). The potency of cell growth inhibition for each compound was determined as an $IC_{50}$ value, which was defined as the concentration that caused a 50 % decrease in cell growth.

### Cell toxicity

To estimate the impact of the compounds on the cellular viability of noncancerous cell lines, human umbilical vein endothelial cells (HUVEC) were used. Cells were seeded on 96-well microplates at a density of $1 \times 10^5$ cells/mL (100 μL medium/well) and were incubated for 12–24 h. Then the culture medium supernatant was removed from the wells, and the cells were treated with 200 μL of various concentrations of test compound for 48 h. After treatment of test compounds, the cytotoxicity was measured using the MTT assay. The $CC_{50}$ value (50 % cytotoxicity concentration in HUVEC cells) was recorded.

### Statistical analysis

In this study, the $IC_{50}$ values were calculated using nonlinear regression model implemented in GraphPad Prism version 6.0. Statistical analysis was conducted to a one-way analysis of variance (ANOVA) and Dunnett's test with $p < 0.05$ versus blank control using IBM SPSS Statistics 20 software package. Results are expressed as means ± standard deviation (SD) of three parallel experimental measurements.

## Results and discussion

### In vitro antioxidant activities

3,4-Dihydroxybenzoic acid (**1**) and its hydroxybenzamide derivatives were evaluated for their antioxidant activities

by DPPH radical scavenging and reducing power. The DPPH radical scavenging activities of compounds **1** and four derivatives increased in a concentration-dependent manner (Fig. 1), and the $IC_{50}$ values of the test compounds for DPPH radical scavenging are shown in Table 1. Although compound **WF** showed an $IC_{50}$ value of 13.57 μM (the corresponding value for ascorbic acid was 17.87 μM), the DPPH scavenging efficiencies of the test compounds were ordered as follows: ascorbic acid > **WF** ≈ **WL** > **WH** ≈ **WN** > **1**. Comparisons between compound **1** and its hydroxybenzamide derivatives showed that carboxyl amidation increased the efficiency. The results illustrate that the amide groups contributed to hydroxylation and the high reactivity of the hydroxyl radical. The kinetic behavior of DPPH radical scavenging by compounds **1** and four derivatives is shown in Fig. 2. In contrast to compound **1**, the steady state was rapidly reached after approximately 5 min for its hydroxybenzamide derivatives. However, the kinetics of compound **1** did not reach steady state during the 40 min in this study, in agreement with prior studies stating that its slower kinetic process lasted 1–6 h (Brand-Williams et al. 1995).

We further evaluate the antioxidant activities of test compounds by the reducing power assay. As shown in Fig. 3, the reducing potential of the test compounds was observed to be in the order **WL** > **WF** > **1** > **WN** > **WH** > ascorbic acid. The efficiency of compounds **1** and four derivatives increased in a dose-dependent manner. In terms of reducing power, compound **1** and its hydroxybenzamide derivatives were more effective hydrogen donors than that of ascorbic acid. The electrons that compounds **1** and four derivatives donated could delay or prevent the oxidation of oxidizable substrates. The high efficiency revealed that the ortho-diphenolic structure was an effective hydrogen donor, in accordance with the

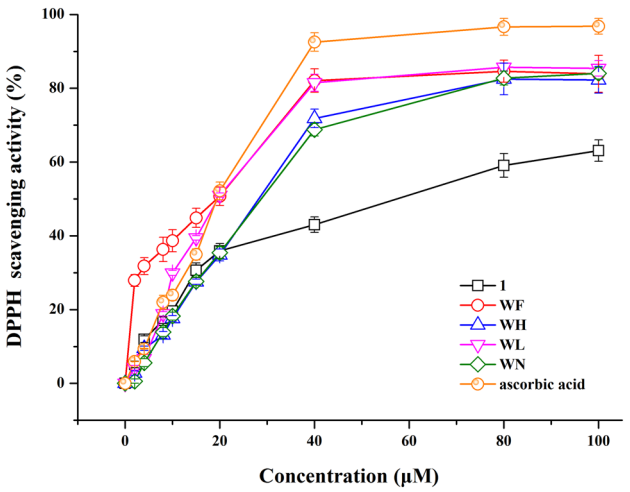



**Fig. 1** DPPH radical scavenging activities of the hydroxybenzamide derivatives and of ascorbic acid

**Table 1** $IC_{50}$ values for the DPPH radical scavenging activity of compounds **1** and four derivatives[a]

| Compound | $IC_{50}$ ± SD (μM) |
|---|---|
| **1** | 50.29 ± 1.03 |
| **WF** | 13.57 ± 1.08 |
| **WH** | 27.28 ± 1.03 |
| **WL** | 19.29 ± 1.03 |
| **WN** | 27.52 ± 1.02 |
| Ascorbic acid[b] | 17.87 ± 1.03 |

[a] Values are means ± SD ($n = 3$)

[b] Positive control

stabilization of the phenoxyl radical by hydrogen bonding or regeneration of another diphenol, as described by Pokorny (Chan 1987) and Cuvelier et al. (1992). Therefore, the above two antioxidant evaluations indicated that the synthesized compounds **1** and four derivatives can be used as the novel superior antioxidants.

## Inhibition of thrombin activity

Since the amido providers *p*-aminomethylbenzoic acid, tranexamic acid, and 6-aminocaproic acid can exhibit a hemostatic effect via the antifibrinolytic process, thus, we investigated the anti-thrombin effects of compounds **1** and its hydroxybenzamide derivatives, and the $IC_{50}$ values are shown in Table 2. Compounds **1**, **WH**, and **WN** exhibited strong inhibitory activity against thrombin-catalyzed hydrolysis of the *p*-nitroanilide tripeptide substrate (Chromozym-TH). As shown in Fig. 4, compounds **WF** and **WL** displayed weak inhibition of thrombin, even at the maximal test concentrations. Compared with all the test compounds, positive control hirudin (a polypeptide) showed the most potent activity, but the oral dosage forms of hirudin was poorly absorbed in body. Thus, small molecule thrombin inhibitors have more superiority in absorption and release in therapeutic process. We further explore the inhibition mechanism of thrombin for the compounds **1**, **WH**, and **WN.** The solutions of compounds **1** at higher concentrations was acidic (pH ≈ 3–4), while the pH values of its hydroxybenzamide derivatives were between 6 and 7. When pH of compound **1** is adjusted to 7 at each concentration, it just exhibited the weak inhibition of thrombin activity at 10 mg/mL (inhibition rate was 45 %). However, the inhibition of thrombin activity was almost unchanged below 5 mg/mL (shown in SI-9). Thus, the loss of thrombin activity may be due to both of the self-property of compound **1** and its low pH value at the high concentration. Among all of hydroxybenzamide derivatives, only **WH** and **WN** indicated grossly inhibitory activity against thrombin in vitro assays. The results demonstrated that vicinal dihydroxyl groups at the benzene ring illustrated a

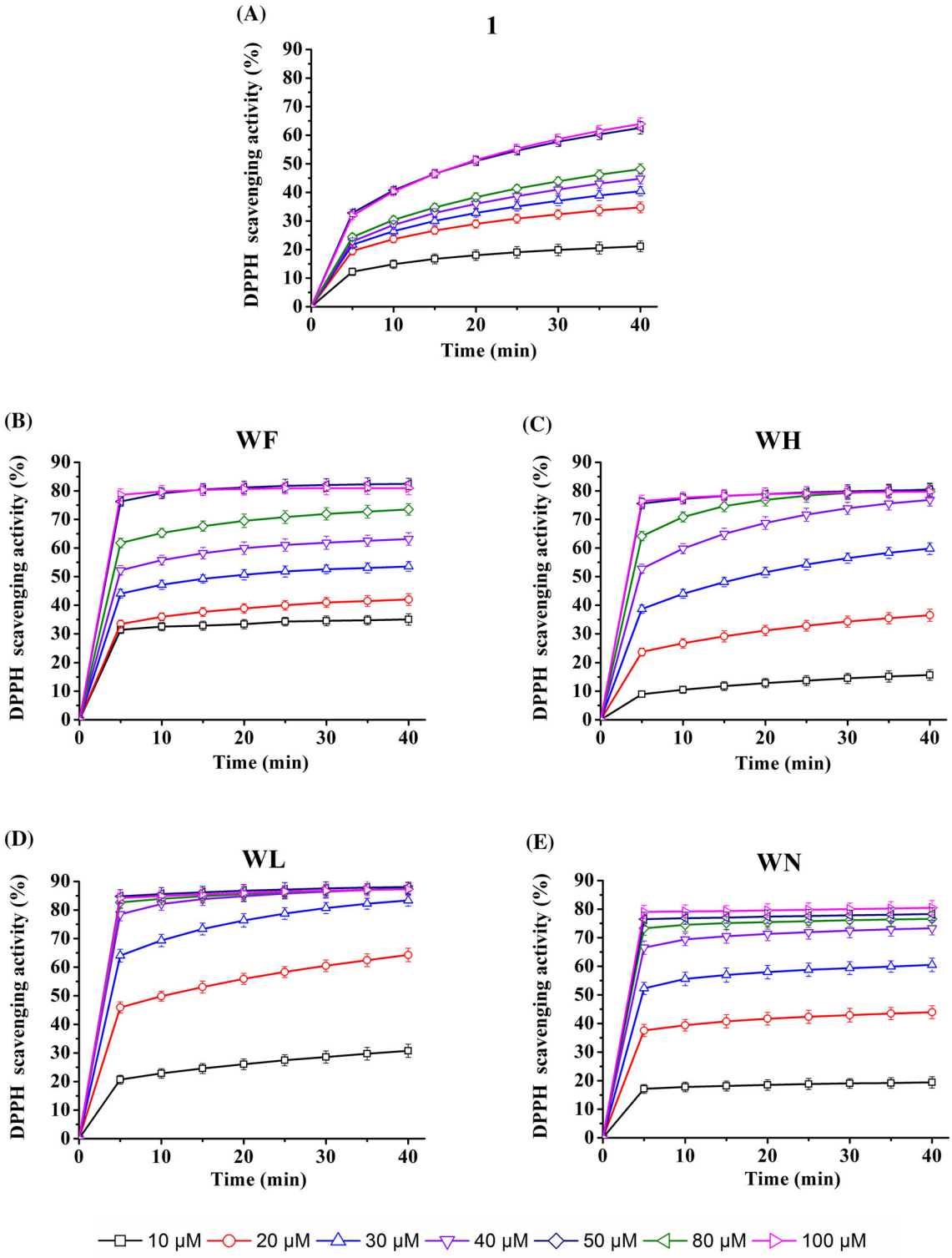



**Fig. 2** The kinetics curves of DPPH radical scavenging by compounds **1** and four derivatives

strong influence on inhibition (Dong et al. 1998). In the compounds **1**, **WH**, and **WN**, the derivative **WN** indicated the best thrombin inhibition ability, we speculate that the superior effectiveness of **WN** compared with both **1** and the other three derivatives was likely caused by its sulfonic group. The above results demonstrate the strong potential application of compounds **1** and hydroxybenzamide derivatives **WH**, **WN** as antithrombotic agents or prodrugs.

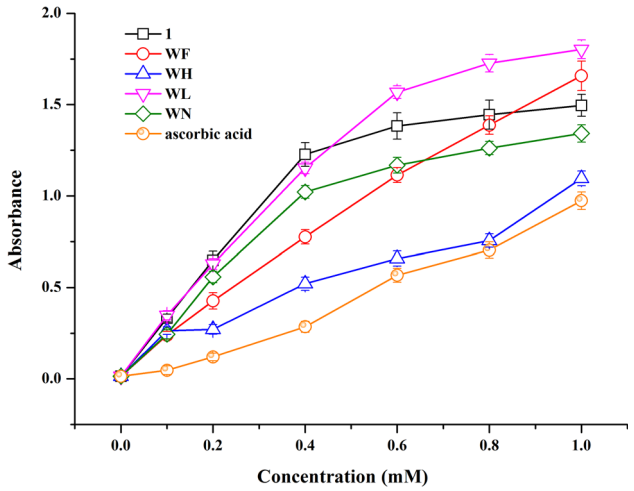



**Fig. 3** Reducing power of the hydroxybenzamide derivatives and of ascorbic acid

**Table 2** $IC_{50}$ values for the inhibition of thrombin activity by compounds **1** and four derivatives[a]

| Compound | $IC_{50} \pm SD$ (mM) |
|---|---|
| **1** | 29.26 ± 0.19 |
| **WF** | –[b] |
| **WH** | 16.43 ± 0.20 |
| **WL** | – |
| **WN** | 6.89 ± 0.08 |
| Hirudin[c] | 0.00002.95 ± 0.00 |

[a] Values are means ± SD ($n = 3$)

[b] No $IC_{50}$ can be obtained

[c] Positive control

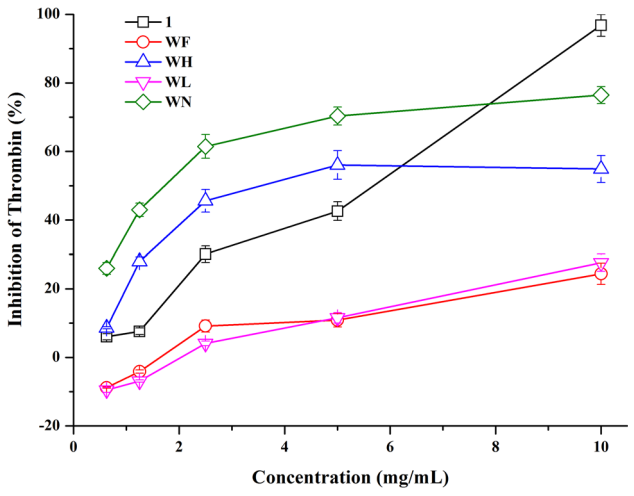



**Fig. 4** Effects of the hydroxybenzamide derivatives on inhibitory activity against thrombin

## In vitro growth-inhibition assay

We further research the anticancer activity of compounds **1** and its hydroxybenzamide derivatives for the previous report that compound **1** was demonstrated to possess anticancer properties. As presented in Fig. 5, growth inhibition by test compounds were determined using MTT assays with human ovarian carcinoma A2780 cells, human breast cancer MCF-7 cells, and human chronic myelogenous leukemia K562 cells. Compounds **1**, **WL**, and **WN** showed cytotoxicity toward all three cancer cell lines, while compound **WF** only had a cytotoxic effect on K562 cells and **WH** showed the growth-inhibitory effects for both A2780 and MCF-7 cells. These results can be ascribed to the difference of the length of the carbon chain, the unsaturated/saturated status of the hexatomic ring between the dihydroxybenzamide group and the terminal carboxylic group (sulfonic group), which would have the influence on the antitumor activity of each derivative. For the A2780 cells, the anticancer effectiveness of the compounds **1** and its derivatives were ordered as follows: **WH** > **WL** > **WN** > **1** > **WF**, and that of sequence for MCF-7 cells was **WL** > **1** ≈ **WN** > **WH** ≈ **WF**. Four derivatives exhibited the similar anticancer effectiveness for the K562 cells, which were better than that of compound **1**. We further calculated the $CC_{50}$ and $IC_{50}$ values of the test compounds and the results are listed in Table 3. Compounds **1** and four derivatives for their catechol structure showed greater cytotoxic activity. These results confirmed that growth-inhibitory properties are affected by the number of ring substituent hydroxyl groups (Fiuza et al. 2004; Gomes et al. 2003). Although the compounds **1** and its derivatives displayed a weaker antitumor effect towards all three cancer cell lines than doxorubicin, their low toxicity toward noncancerous cells meets a vital requirement for anticancer agents. Therefore, compounds **1** and its derivatives have the well anticancer activity and excellent biocompatibility, which have the potential application in the prevention and treatment of cancer.

In summary, 3,4-dihydroxybenzoic acid (**1**) and its hydroxybenzamide derivatives were synthesized under mild reaction conditions in this study. Compounds **WF** and **WN** were obtained for the first time. The antioxidant, thrombin-inhibitory, and anticancer activities of hydroxybenzamide derivatives are prominently affected by the presence of ring substituent hydroxyl groups. Through the various standard evaluation methods, hydroxybenzamide derivatives demonstrate superior multifunctional activities such as antioxidation, free-radical scavenge, inhibition of thrombin, and anticancer. Moreover, this series of derivatives shows little harmful effects on normal cells, indicating

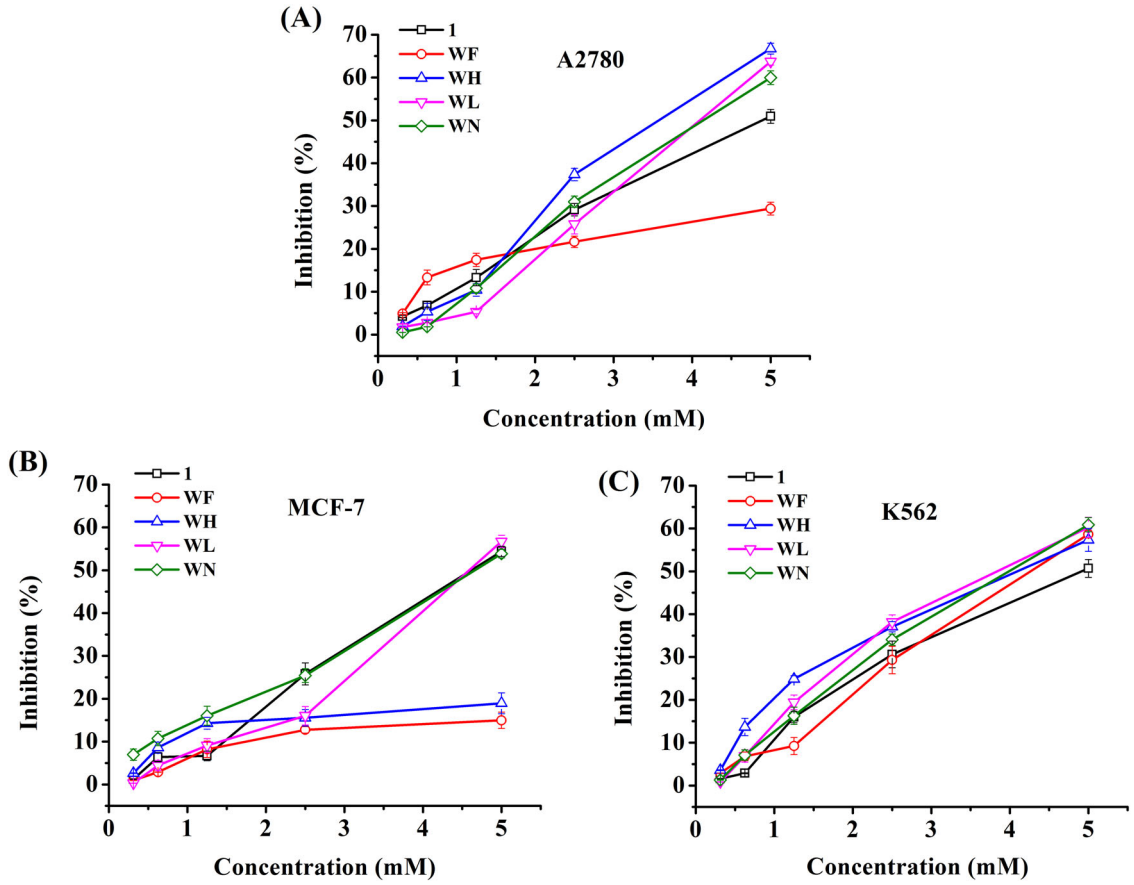



**Fig. 5** Inhibitory effects of the hydroxybenzamide derivatives against human ovarian carcinoma cells (A2780), human breast cancer cells (MCF-7), and human chronic myelogenous leukemia cells (K562), as determined by the MTT assay

**Table 3** Cytotoxicity in HUVEC cells and inhibitory effects of the test compounds against cancer cell lines expressed as $IC_{50}$ values[a]

| Compound | $CC_{50}^{b}$ ± SD (mM) | $IC_{50}^{c}$ ± SD (mM) | | |
|---|---|---|---|---|
| | | A2780 | MCF-7 | K562 |
| **1** | >10 | 4.90 ± 1.02 | 4.54 ± 1.03 | 4.77 ± 1.04 |
| **WF** | >10 | –[d] | – | 4.13 ± 1.03 |
| **WH** | >10 | 3.41 ± 1.02 | – | 3.85 ± 1.04 |
| **WL** | >10 | 3.93 ± 1.01 | 4.55 ± 1.03 | 3.62 ± 1.03 |
| **WN** | >10 | 3.96 ± 1.01 | 4.88 ± 1.07 | 3.78 ± 1.02 |
| Doxorubicin[e] | 0.02 ± 0.00 | 0.27 ± 0.02 | 0.08 ± 0.03 | 0.24 ± 0.06 |

[a] Values are means ± SD ($n = 3$)

[b] $CC_{50}$ is 50 % cytotoxicity concentration in HUVEC cells

[c] $IC_{50}$ is 50 % inhibitory concentration in three cancer cell lines

[d] No $IC_{50}$ can be obtained

[e] Positive control

the excellent biocompatibility. Our findings suggest that these dihydroxybenzamide derivatives may be considered for disease prevention and pharmaceutical research of cancer, thrombotic diseases, and aging due to their underlying biological activities and multiple functions.

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**Supporting Information** Structural formulae of hydroxybenzamide derivatives.

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