Design, Synthesis and Antioxidant Activity of Chalcone Derivative CMZ-3-5

Cen Xiang, Xiangshun Kong, Feng Gao, Yufan Zhao, Jiang Liu, Yuan Yuan* and Yuou Teng**
China International Science and Technology Cooperation Base of Food Nutrition/Safety and Medicinal Chemistry, Tianjin University of Science and Technology, Tianjin 300457, China.
tyo201485@tust.edu.cn*

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Summary: Excessive accumulation of free radicals can cause a variety of diseases. According to reports, chalcone has the activity of scavenging free radicals. In this study, we synthesized a chalcone derivative CZM-3-5 and evaluated its antioxidant activity using a chemical assessment method (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) assay, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) assay and total reducing antioxidant power (FRAP) assay) and a cell model of oxidative damage induced by hydrogen peroxide (H_2O_2). The chemical evaluation results show that the compound CZM-3-5 has good antioxidant capacity and free radical scavenging ability. MTT analysis showed that the compound has cytoprotective activity in H_2O_2-induced PC12 cells, and the mechanism may be related to the cytoprotective substance superoxide dismutase (SOD) is related to the level of glutathione (GSH). Overall, our findings indicate that compound CZM-3-5 has potential antioxidant activity *in vitro*.

Keywords: Antioxidant; Chalcone; PC-12 cells; SOD; GSH

Introduction

Free radicals play an important role in organisms [1, 2]. Too much free radicals in the body can cause cell damage and death, and many diseases are related to it, such as cancer [3], stroke [4], myocardial infarction [5], diabetes [6]. The human body has various mechanisms to defend against and repair damage caused by free radicals, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase [7]. Furthermore, Antioxidants such as vitamin A, vitamin C (VC) and vitamin E[8] and polyphenols[9] play a key role in these defense mechanisms.

Flavonoids are widely distributed in nature, such as fruits, vegetable, tea, wine and so on [10]. Flavonoids have a variety of bioactivities, examples include antitumor, antioxidant, antiviral, and anti-inflammatory effects [11-18]. Chalcone is an important skeleton structure with biological compounds and is reported to have a wide range of biological activities [19-22].

The oxidative damage model of PC12 cells induced by H_2O_2 can be used to evaluate the antioxidant properties *in vitro* [23]. H_2O_2 is the main reactive oxygen species (ROS) with high cell membrane permeability, which can cause cell membrane lipid peroxidation and intracellular DNA damage [24]. It is commonly used as a toxic substance that mimics oxidative stress-induced damage *in vitro*. PC12 cell line is derived from the rat adrenal medulla pheochromocytoma and is the most commonly used cell model system for studying nerves and neurochemistry [25]. The chemical antioxidant evaluation method is a fast, convenient and highly reproducible method. Antioxidant capacity can be demonstrated by chemical substances that inhibit or scavenge free radicals, such as scavenging of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [26] (ABTS), 2,2-diphenyl-1-picrylhydrazyl [27] (DPPH) and total reducing antioxidant power [28] (FRAP).

In this study, we have synthesized and investigate antioxidant capacity of CZM-3-5. The oxidative damage model of PC-12 cells was established and the compound CZM-3-5 was subjected to perform further preliminary mechanism study and *in vitro* antioxidant activity analysis.

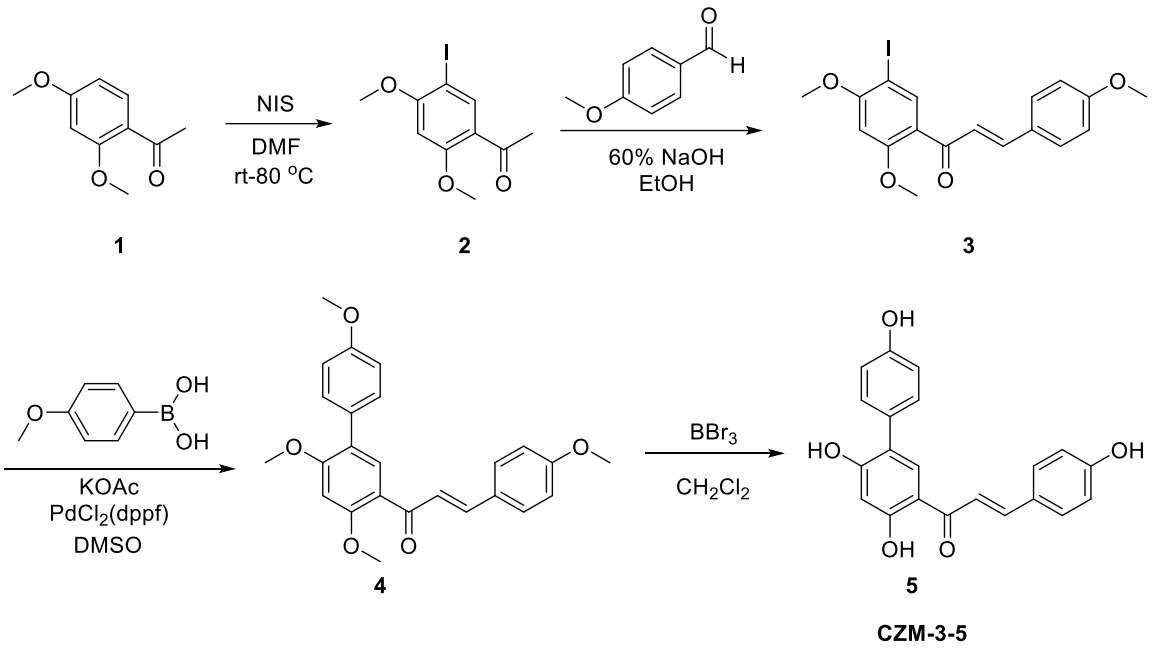
Experimental

Chemistry

1-(5-iodo-2,4-dimethoxyphenyl)ethan-1-one **(2)**

To a stirred solution of 1-(2,4-dimethoxyphenyl)ethan-1-one (1) (5.0 g, 27.75 mmol) in N,N-dimethylformamide (15 mL) was added NIS (7.5 g, 33.30 mmol). The reaction mixture was heated to 80℃ for 6 h. After cooling to room-temperature, added the reaction mixture to 50 mL water and then extracted by EtOAc (50 mL×3). The combined organic layer was dried on Na_2SO_4 and then concentrated to obtain residues, which were purified by column chromatography (PE: EtOAc = 7 : 1) to give compound 2 (6.4 g, 75%).

*To whom all correspondence should be addressed.



Scheme-1: Synthetic route for target compounds CZM-3-5.

^{1}H NMR (400MHz, $CDCl_3$): δ 2.56 (s, 3H), 3.940 (s, 3H), 3.944 (s, 3H), 6.39 (s, 1H), 8.32 (s, 1H).

(E)-1-(5-iodo-2,4-dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one **(3)**

To a stirred solution of 1-(5-iodo-2,4-dimethoxyphenyl)ethan-1-one (2) (2.0 g, 6.53 mmol) in EtOH (10 mL) was added 4-methoxybenzaldehyde (935 mg, 6.86 mmol). The reaction mixture was cooled to 0℃ and 60 % aq. NaOH (5 mL) was added. Then the reaction mixture was warmed to room temperature and stirred for 12 h. The reaction mixture was diluted with water (50mL) and extracted via EtOAc (50mL×3). The combined organic layers were dried over Na_2SO_4 and then concentrated to give a residue which was purified by column chromatography (PE : EtOAc = 15 : 1) to give compound 3 (2.5 g, 90%).

^{1}H NMR (400MHz, $CDCl_3$):δ 3.85 (s, 3H), 3.94 (s, 3H), 3.96 (s,3 H), 6.44 (s, 1H),6.91(d, J = 8.8 Hz, 2H), 7.32 (d, J = 15.6 Hz, 1H), 7.55 (d, J = 8.8 Hz, 2H), 7.65 (d, J = 15.6 Hz, 1H), 8.13 (s, 1H).

(E)-3-(4-methoxyphenyl)-1-(4,4',6-trimethoxy-[1,1'-biphenyl]-3-yl)prop-2-en-1-one **(4)**

A stirred solution of (*E*)-1-(5-iodo-2,4-dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (3) (200 mg, 0.47 mmol) in DMSO (1.5 mL) were added (4-methoxyphenyl)boronic acid (77mg, 0.56 mmol), KOAc (93 mg, 0.94 mmol) and $PdCl_2$(dppf) (17 mg, 0.023 mmol). The reaction mixture was heated by microwave under argon for 1.5 hours at 90℃.The reaction mixture was diluted by water (50mL) and extracted with EtOAc (50mL×3). The combined organic layer was dried by Na_2SO_4 and then concentrated to obtain the residue, which was purified by column chromatography (PE: EtOAc = 8:1) to obtain compound 3 (110mg, 65%).

^{1}H NMR (400MHz, $CDCl_3$): δ 2.38 (s, 3H), 3.85 (s, 3H), 3.90 (s, 3H), 3.99 (s, 3H), 6.57 (s, 1H), 6.91 (d, J = 8.4 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 7.39~7.43 (m, 3H), 7.56 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 15.6Hz, 1H), 7.76 (s, 1H).

(E)-3-(4-hydroxyphenyl)-1-(4,4',6-trihydroxy-[1,1'-biphenyl]-3-yl)prop-2-en-1-one (CZM-3-5)

Boron tribromide (100 mg, 0.4 mmol) was added to a stirred solution of (*E*)-3-(4-methoxyphenyl)-1-(4,4',6-trimethoxy-[1,1'-biphenyl]-3-yl)prop-2-en-1-one (4) (40 mg, 0.1 mmol) in

anhydrous DCM (5 mL) under -78℃ for 30 min. The reaction mixture was then slowly warmed to room-temperature for 12 hours. Again, the reaction mixture was cooled to 0℃, and the excess ice-water was added to the reaction mixture under stirring for 10 min. The reaction solvent was removed by decompression to obtain the suspension, which was then filtered and washed by water. After drying, the compound CZM-3-5 (34mg, 98%) was obtained.

^{1}H NMR (400MHz, $CDCl_3$): δ 6.44 (s, 1H), 6.79-6.81 (m, 4H), 7.35 (d, J = 8.8 Hz, 2H), 7.77-7.81 (m, 4H), 8.05 (s, 1H), 9.39 (s, 1H), 10.25 (s, 1H), 13.58 (s, 1H).

Cell culture

PC12 cell line was obtained from the Shanghai Institutes of Biological Sciences (Shanghai, China). For PC12 cells, use RIMP1640 culture solution (containing 1% penicillin and streptomycin mixture and 10% fetal bovine serum) in 37 ℃, 95% relative humidity, 5% CO_2 concentration incubator Medium cultivation.

Chalcone derivatives assay on H_2O_2-damaged PC-12 cells

H_2O_2-induced oxidative-damage PC-12 cell model was established as a measure of the antioxidant activity of CZM-3-5. PC12 cell model induced by H_2O_2 was established and cell viability was assessed by MTT assay as a measure of antioxidant activity of CZM-3-5, VC, a known potent antioxidant, was used as a positive control in MTT analysis. CZM-3-5 and VC were pre-dissolved in DMSO. PC12 cells were prepared in 90 μL medium with 5×10^5 cells/well in 96-well plate. After incubation for 24 h, 0.5 μL of the CZM-3-5 (3, 10, 30, 100 μmol/L), VC (200 μmol/L) or DMSO were added to each well and incubated for 0.5 h. The cells were incubated with H_2O_2 in RIMP1640 culture solution (10 μL, 1 mmol/L) for 2 hours to induce cell damage. After 2 h, each well were photographed under an inverted microscope (40×) for observation of morphological changes. After the microscopic examination is completed, 20 μL 5 mg/mL MTT solution were added to each well. After incubation for 4 h, Discard the medium, add 100 μL of DMSO, incubate for 10 min, and take readings at 490 and 630 nm. For each treatment, mean cell viability was calculated from three separate experiments. Cell viability was specified as 100% in DMSO-treated controls.

Determination of SOD and GSH

We tested the content of SOD and GSH in cells when the cells were exposed to VC (100 μmol/L) or CZM-3-5(200 μmol/L). The method is the same as 2.6. Finally, collect the cells and break them with 4% chaps. superoxide dismutase (SOD) activity and glutathione (GSH) content were determined in PC-12 cells by kits (Nanjing jiancheng Bioengineering Insititute, Nanjing, China) according to the manufacturer's instructions.

Evaluation of antioxidant capacity by chemical method

FRAP assay

The principle of FRAP[29] is that in solution with a low PH value, Fe^{3+}-TPTZ is reduced to colored Fe^{2+}-TPTZ by antioxidants. The compound CZM-3-5 was diluted to different concentrations (0.06、0.2、0.6、2、6、20、60 mmol/L) for the experiment. In the experimental system, two synthetic antioxidants of BHA and BHT were added as positive controls. The VC standard was diluted to different concentrations (100-600 g/L) for the experiment.

$ABTS^{\bullet+}$ assay

$ABTS^{\bullet+}$ assay [30] is a widely used method for the determination of antioxidant activity. The capacity of antioxidants to scavenging $ABTS^{\bullet+}$ can be evaluated with absorbance at 414 nm or 734 nm. The compound CZM-3-5 was diluted to different concentrations (0.06、0.2、0.6、2、6、20、60 mmol/L) for the experiment. In the experimental system, two synthetic antioxidants of BHA、BHT and a natural antioxidant of VC were added as positive controls.

$DPPH^{\bullet}$ assay

It can act as an antioxidant if the sample can clear $DPPH^{\bullet}$. Therefore, the antioxidant capacity of the compounds under test can be measured by the capacity of scavenging $DPPH^{\bullet}$ [31]. The stability of the experimental system was verified by adding VC as positive control in each experiment. The compound CZM-3-5 was diluted to different concentrations (0.06、0.2、0.6、2、6、20、60 mmol/L) for the experiment. In the experimental system, two synthetic antioxidants of BHA and BHT were added as positive controls.

Statistical analysis

All data were expressed as mean ± S.D. The

results were analyzed by one-way analysis of variance (ANOVA) and significant differences were determined by post hoc Tukey's test using Graph Pad Prism 7.0. *p* value <0.05 was considered as significant.

Results and Discussion

Antioxidant capacity of compound CZM-3-5 in vitro

Table-1: FRAP and $ABTS^{\bullet+}$, $DPPH^{\bullet}$ scavenging activities of CZM-3-5.

Test compound	Equivalent amount of Vc (mg/mmol)	Scavenging activity (EC_{50} mmol/L)	
	FRAP	ABTS	DPPH
BHA	90.11±12.53	1.41±0.44	28.30±6.69
BHT	221.30±15.57	7.68±6.47	5.36±1.23
VC	-	0.52±0.03	0.71±0.10
CZM-3-5	215.60±13.09	51.41±12.16	9.46±1.36

Exogenous free radical producing agent is the key of the determination method *in vitro*, and its antioxidant activity is determined by the ability of inhibitory or scavenging the free radical initiator with stable chemical property. In this FRAP assay (Table 1), a recognized synthetic antioxidants, BHA, was employed as the comparison group to BHT and CZM-3-5, which displayed the best antioxidant activity. The equivalent amount of VC of CZM-3-5 is 215.60 mg/mmol which is higher than BHA. In the $ABTS^{\bullet+}$assay, EC_{50} of VC, BHA、BHT、VC、CZM-3-5 were 1.41、7.68、0.52、51.41mmol/L, respectively. As can be seen from the table, VC has the best ability to scavenge $ABTS^{\bullet+}$, and CZM-3-5 is the weakest. In $DPPH^{\bullet}$ scavenging test, the natural antioxidant VC has the best ability to clear $DPPH^{\bullet}$, the EC_{50}.was 5.36 mmol/L. EC_{50} of CZM-3-5 was 9.46 mmol/L, lower than BHT (28.30 mmol/L), but better than BHA (5.36 mmol/L).

The cell viability of compound CZM-3-5 is 60.61%. Although its viability was lower than that in the VC group, it was higher than that in the control group. Consequently, we selected CZM-3-5 for further study (Figure 1A). The results (Figure 1B) showed that treatment with H_2O_2 (100 μM) for 2h obviously increased the cell oxidative stress injury. Morphological changes in CZM-3-5 treated PC-12 cells suggested that it significantly protected them against H_2O_2-induced cell death.

*Evaluation of antioxidant activity of CZM-***3-5**

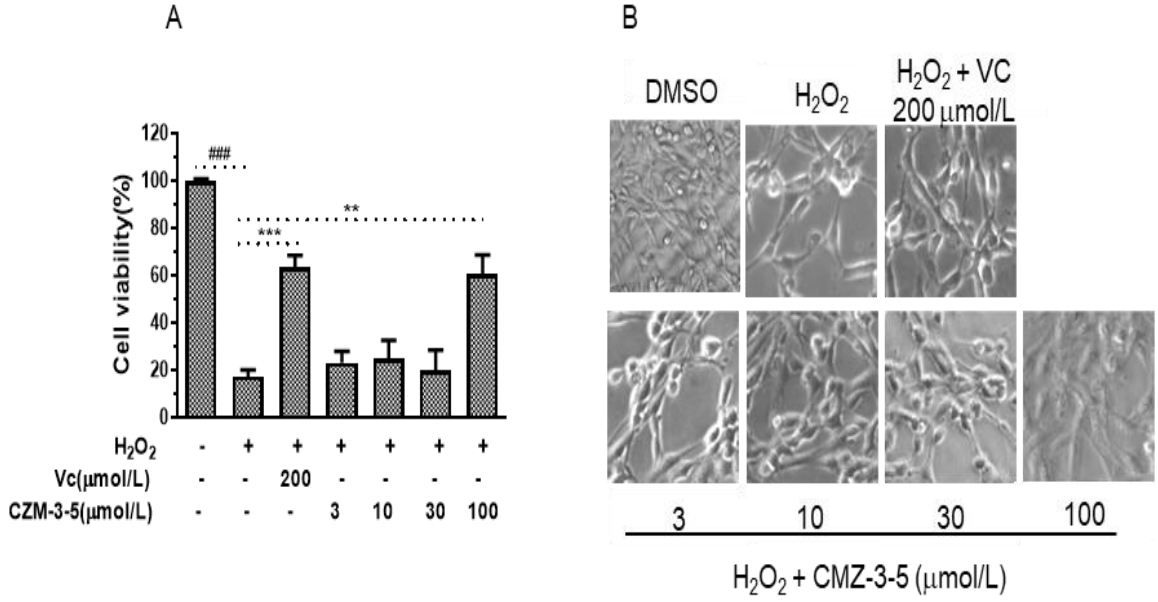


Fig. 1: Protect ability of chalcone in oxidative-damaged PC-12 cell model. (A) Cells were pre-protected with various concentrations of CZM-3-5 for 0.5h. DMSO group without H_2O_2 served as a blank control group and it was assigned a cell viability value of 100%. (B) Morphological changes in CZM-3-5 treated PC-12 cells. $^{\#\#}P<0.01$ compared to control cells; $^{**}P<0.01$ compared to H_2O_2 treated cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

*SOD and GSH content in PC-12 cells treated with CZM-***3-5**

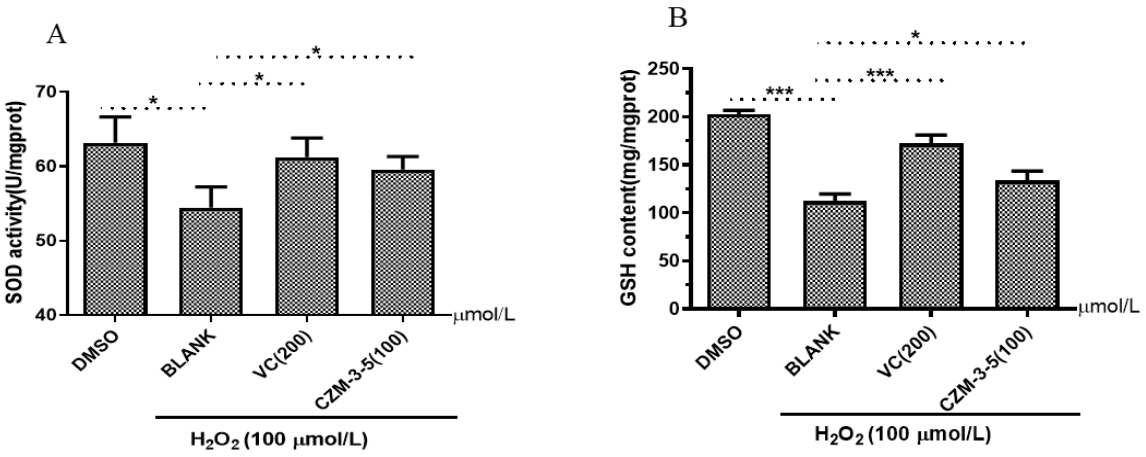


Fig. 2: Content of SOD (A) and GSH (B) in PC-12 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The results showed, the SOD content of the positive control group VC was 61.241 U/mgprot., which was slightly lower than that of the blank group, but higher than that of the negative control group with H_2O_2. Therefore, the experimental system was normal. As shown in Fig 2A, pretreat with CZM-3-5 markedly increased the SOD level from 53.79 to 58.57 U/mgprot.

The GSH content of the positive control group VC was 172.31 mg/mgprot., which was slightly lower than that of the blank group, but higher than that of the negative control group with H_2O_2. Therefore, the experimental system was normal. Compound CZM-3-5 protected the PC-12 cells against H_2O_2 oxidation through increase the GSH level by 133.59 mg/mgprot.

A large number of studies have shown that chalcone and its derivatives exhibited positive effects on various diseases' therapy, such as neurodegenerative diseases [32], cardiovascular diseases [33], liver injury [34], cancers [35], etc. The working mechanism of chalcone and its derivatives holding positive effects on above diseases are largely related to their outstanding antioxidant activities [36]. In this study, a derivative of chalcone, CZM-3-5, was synthesized, and subsequently, the antioxidant capacity was also evaluated, via both chemical evaluation method and H_2O_2-induced PC12 cell model.

The FRAP assay results revealed, *in vitro* antioxidant activity of compound CZM-3-5 is better than the commonly used food antioxidant BHT; in the $ABTS^{\bullet+}$ radical scavenging activity assay, the CZM-3-5 radical scavenging rate is compared to the positive control VC BHT and BHA are poor; however, compound CZM-3-5 is better than BHA in removing DPPH free radicals. In addition, compound CZM-3-5 can reverse the decrease in cell viability caused by H_2O_2 and significantly increase cell viability. This study indicates that compound CZM-3-5 has potential antioxidant activity *in vitro*.

Antioxidant capacity can be reflected by free radicals scavenging [37], which is one of the main antioxidant mechanisms to inhibit the chain reaction of lipid peroxidation. In our study, the compound CZM-3-5 showed effective antioxidant properties to scavenge free radicals (Table-1). The results of this experiment suggest that our compound CZM-3-5 may be considered a promising compound for the prevention and treatment of chronic diseases caused by oxidative stress. Based on this analysis, we hypothesized that compound CZM-3-5 may prevent cellular-level damage caused by oxidative stress. To test our hypothesis, we next used the H_2O_2-induced damage model of PC12 cells to study the antioxidant capacity of compound CZM-3-5. As we expected, compound CZM-3-5 has good antioxidant activity against H_2O_2- induced PC12 cell damage.

Excessive ROS exposure will disrupt the

redox homeostasis, leading to oxidative stress, damage to important organelles and biological molecules, such as DNA and proteins, mediated by ROS, as well as damage related to carcinogenesis, diabetes, neurodegeneration and aging [38]. In the body, SOD and GSH are important protective substances against oxidative stress disorder in the body, and they can effectively eliminate the excessive ROS in the body [39]. To study the mechanism of action of compound CZM-3-5, the levels of SOD and GSH in the H_2O_2-induced PC12 cell damage were evaluated. We found that the mechanism of compound CZM-3-5 was related to the increase of SOD and GSH contents in cells.

Although the compound CZM-3-5 is not as good as VC in antioxidative activity, VC is easily oxidized and deteriorated in the air, so that VC's medicinal properties are not high. Compound CZM-3-5 may have further modification in antioxidant activity.

Conclusions

In this study, CZM-3-5, a chalcone derivative, was designed and synthesized. In the test of antioxidant capacity at chemical level, compound CZM-3-5 showed good antioxidant capacity and free radical scavenging ability. In the cell level test, compound CZM-3-5 also showed good antioxidant capacity in H_2O_2-induced PC12 cell model, and its mechanism of action were related to the increase of SOD and GSH contents in cells. In summary, compound CZM-3-5 is a potential lead compound for antioxidant.

Conflicts of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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