The Synthesis of a Novel Chalcone and Evaluation for Anti-free Radical Activity and Antagonizing the Learning Impairments in Alzheimer's Model

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Key Words

Alzheimer's • Free radical injury • Polyphenols • Hydroxyl-substituted chalcones

Abstract

We synthesized a new chalcone (4,2'-dihydroxy-3-methoxy-5-bromine chalcone; C) and structurally identified it via infrared spectrometry (IR), ¹H-NMR, mass spectrometry (MS) and element analysis (EA). C was confirmed to be highly potent in scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) and OH free radicals *in vitro*. Tests of anti-free radical activity in response to oxidative stress in mice revealed that C could elevate glutathione peroxidase (GSH-PX) and super oxide dismutase (SOD) levels and lower malonaldehyde (MDA) level in a free-radical-injured scopolamine-induced Alzheimer's model. Further behavioral tests with the Morris water maze showed that C could antagonize the learning impairments in the Alzheimer's model, which suggests that C has a potential role in Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common form of neurodegenerative disease associated with dementia in elderly people. Although the initiating events are still unknown, AD, at least in its sporadic form, results from a combination of genetic risk factors and different epigenetic events. A growing body of evidence suggests that AD pathogenesis involves an imbalance between free radical formation and destruction [1-5]. This concept originally derived from the free radical hypothesis of aging, with age-related accumulation of free radicals resulting in damaged cell components. That age is a key risk factor in AD provides support for this hypothesis [6, 7]. A long list of surrogate markers, including lipids, DNA, and protein oxidation, for oxidant stress-mediated injury, have been reported as being elevated in the AD brain. Moreover, epidemiologic studies show that dietary intake of natural or synthetic products such as vitamin E has a putative antioxidant effect in reducing the risk of AD [8].

In this study, we aimed to synthesize a new polyphenol-3-methoxy-4,2'-dihydroxy-5-bromo chalcone (C) and test its anti-free-radical activity *in vivo* and *in vitro* to find a new structure with potential for AD

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treatment. We designed this structure because of the following:

1) C, a sort of hydroxyl chalcone, is a vinylog of exifone (Fig. 1A), an anti-AD drug that treats cognitive decline associated with aging and corrects memory dysfunction [9]. C was presumed to have chemical effects similar to those of exifone because of almost identical structure and vinylog.

2) Chalcones (Fig. 1B) constitute an important class of natural products belonging to the flavonoid family, which have a wide spectrum of biological activities, including antibacterial, antifungal, anti-inflammatory, antimicrobial, antitumor, insect antifeedant and antimutagenic activities [10]. Hydroxyl chalcones embrace hydroxyl substitutions, one of the key groups to greatly enhance the antioxidant activity of chalcones, mainly because of their easy conversion to phenoxy radicals through the hydrogen atom transfer mechanism. These chalcones were presumed to possess anti-free radical activities.

3) The structure of C (Fig. 1C) has a feruloyl from ferulic acid, which was confirmed in many reports to be highly effective in anti-free radical activity at low concentration because of an extremely active phenol-hydroxyl [11]. As well, the Structure-activity relationships of trans-4-hydroxyl-phenylacrylic acid illustrated that a Br o-substitution of the 4-hydroxyl might elevate the activity of anti-free radicals as compared with substitution of trans-ferulic acid (trans-4-hydroxyl-3-methoxyl-phenylacrylic acid) in our previous study [12]. C might have anti-free radical activity similar to that of ferulic acid because of structural similarity.

Therefore, we designed the C structure for synthesis in our lab and for testing in terms of activities in scavenging DPPH and OH free radicals *in vitro*. Following that, we treated mice with C to test SOD, MDA and GSH-PX levels in free radical-injured scopolamine-induced AD models to assess the potential of C in anti-AD treatment.

Materials and Methods

Chemistry

Synthesis of C. C was successfully synthesized via Claisen-Schmidt condensation using piperidine as a catalyst (Scheme 1). All synthetic reagents were from Shanghai Jingchun Reagents Co. A 250-ml pear-shaped flask, with a mixture of 4,2'-dihydroxyl-3-methoxyl-5-Br-benzaldehyde (23.1 g), 2-hydroxyl-acetophenone (22.4 g) and piperidine (10 mL), was put into an oil bath (160°C) (equipped with an effective magnetic stirring and a reflux condenser) immediately. After 10 min, the mixture

Scheme 1. The synthesis of the polyphenol-3-methoxy-4,2'-dihydroxy-5-bromo chalcone (C).

turned into a dark red sticky liquid and was poured, with ethanol absolute-rinse for 3 times, into a 500-ml beaker with 10% sufficient NaOH in an ice-bath. With vigorous stirring, the mixture turned into an orange-red, non-sticky and uniform solution. Under the same conditions, saturated hydrochloric acid was added into the beaker until the pH reached 2-3, then ethanol-absolute was added to form a mixture of a turbid yellow solution and a dark-brown floating oily substance separated from each other. The mixture was cooled and kept at 4-5°C for one day. The yellow solid crystallized solution was filtered with adequate ethanol-absolute washing until it appeared as bright-yellow crystal and was dried. The product weighed 25.7 g at a yield of 73.6%.

Pharmacology

Animal Selection. Kunming(KM) mice weighing 22-25 g were maintained by Animal Center of Southern Medical University (Guangzhou, China). All animal work was performed according to the international animal welfare guidelines, and protocols were approved by Shantou University Medical College Institutional Animal Care and Use Committee.

Mouse Hepatocyte Isolation. Hepatocytes were isolated from male mice using the two-step collagenase perfusion as previously described by Seglen, with minor modifications [13]. In brief, animals were anesthetized by intraperitoneal injection with pentobarbital (Sigma) (50 mg/kg) and the livers were perfused with 0.5mM EGTA in Ca, Mg-free Hank's balanced-salt solution (HBSS(-)) from the portal vein for approximately 5 min., and then perfused with 0.05% collagenase + trypsin inhibitor (Sigma) in Hank's balanced-salt solution (HBSS) for 5min. The dispersed cells were washed with ice-cold HBSS(-) and suspended in William's E medium supplemented with 10% FBS, 0.1 μM insulin (Sigma), 1 μM dexamethasone (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10 mM HEPES (PH 7.3). Viability of the hepatocytes was determined by trypan blue exclusion and was typically greater than 90%. Finally, the hepatocytes were seeded in 24-well plates and the medium were replaced with William's Media E for Lactate Dehydrogenase (LDH) assay.

Tests of activity of C in scavenging DPPH-free radicals C and Vc (positive control) were prepared as ethanol-absolute solutions of 0.19, 0.38, 0.75, 1.5, 3, 6, and 12 mmol/L. All processes followed procedures in Table 1 (n=8), and all CL (DPPH) values were calculated by Eq. 1. A represents absorption values in 517 nm [14, 15].

Order	Reagents	Nonmodel(μL)	Control(μL)	Sample (μL)
1	Ethanol-absolute	280	240	—
2	DPPH·	—	—	240
3	C	—	40	40
Final volume		280	280	280

Table 1. Process of DPPH-free-radical determination.

Order	Reagents	Nonmodel (μL)	Uninjured (μL)	Injured (μL)	Control (μL)	C (μL)
1	HAc (pH=3.0)	300	300	300	300	300
2	Purified water	180	180	—	180	—
3	Ethanol-absolute	120	—	120	—	—
4	Phen	—	60	60	—	60
5	FeSO ₄	—	60	60	—	60
6	C	—	—	—	120	120
7	H ₂ O ₂	—	—	60	—	60
Final volume		600	600	600	600	600

Table 2. Process of OH-free-radical determination.

Group Name	Number & Sex	d1–d13	d14
control	5♂, 5♀	○	○+△
model	5♂, 5♀	○	○+▲
piracetam	5♂, 5♀	□	□+▲
C (low dose)	5♂, 5♀	■	■+▲
C (medium dose)	5♂, 5♀	◇	◇+▲
C (high dose)	5♂, 5♀	◆	◆+▲

Table 3. Animal treatments concerning the effects of C on markers of oxidative stress. ○, blank CMCNa – suspension per day in corresponding doses after conversion by gavage; □, 500 mg/kg piracetam (in CMCNa – suspension)/day by gavage; ■, 125 mg/kg C (in CMCNa – suspension)/day by gavage; ◇, 250 mg/kg C (in CMCNa – suspension)/day by gavage; ◆, 500 mg/kg C (in CMCNa – suspension)/day by gavage; △, normal saline per day in corresponding doses after conversion by intraperitoneal injection; ▲, 5 mg/kg scopolamine/day by intraperitoneal injection (Notes: Intraperitoneal injections were given 30 min after gavages).

Group Name	Number & Sex	d1–d14	d15 – d21	d22	d23
control	5♂, 5♀	○	○+△+☆	○+△+★	○+△+*
model	5♂, 5♀	○	○+▲+☆	○+▲+★	○+▲+*
piracetam	5♂, 5♀	□	□+▲+☆	□+▲+★	□+▲+*
C (low dose)	5♂, 5♀	■	■+▲+☆	■+▲+★	■+▲+*
C (medium dose)	5♂, 5♀	◇	◇+▲+☆	◇+▲+★	◇+▲+*
C (high dose)	5♂, 5♀	◆	◆+▲+☆	◆+▲+★	◆+▲+*

Table 4. Animal treatments for Morris water maze test. ☆, Spatial navigation test; ★, Spatial exploration test; *, Visible platform test; the others are the same as the footnotes of Table 3 (Notes: Intraperitoneal injections were given 30 min after gavages; water maze test was implemented 30 min after intraperitoneal injections).

$$\text{Eq. 1: CL (DPPH)} = [1 - (A_{\text{control}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100 \%$$

Tests of activity of C in scavenging OH free radicals 0.1% H₂O₂ (29.5 mmol/L, calibrated by potassium permanganate

titration), 7.5 mmol/L ferrous sulfate (newly prepared), 7.5 mmol/L phen (dissolved in ethanol), 0.1 mmol/L acetic acid (pH=3.0), C and Vc (positive control) were prepared as ethanol-absolute

solutions of 0.19, 0.38, 0.75, 1.5, 3, 6, 12 mmol/L. All processes followed procedures in Table 2 (n=8), and all CL (OH) values were calculated by Eq. 2. *A* represents absorption values in 536 nm [16].

$$\text{Eq. 2: CL (OH)} = \frac{[(A_{\text{sample}} - A_{\text{control}}) - (A_{\text{injured}} - A_{\text{blank}})]}{(A_{\text{uninjured}} - A_{\text{injured}})} \times 100\%$$

Tests of C on SOD, MDA and GSH-PX levels on scopolamine-induced Alzheimer's models

SOD, MDA and GSH-PX testing kits were from Nanjing Jiancheng Bioengineering Institute (China). The mice, housed 5 per cage with free access to water and a standard laboratory diet, were subjected to alternate 12-h periods of dark and light (lights on at 6:00 to 18:00) with temperature about 25°C and humidity 40%-60%. Mice were randomly divided into 6 groups for treatment (Table 3), fed 3 times (at 6:00, 12:00 and 18:00) with an equal amount of feed, weighed at 8:00, and treated at 9:00 each day (Table 3). At 30 min after treatment by intraperitoneal injection on day 14, animals were sacrificed, and whole brain tissues were removed. Homogenates were made of 10% (w/v) in cold normal saline, and supernatants were collected immediately after 12000 r/min centrifugation for 15 min and stored at -20°C following the instructions of Nanjing Jiancheng Bioengineering Institute.

Determination of Lactate Dehydrogenase (LDH) Release. Quantification of the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was always performed to evaluate plasma membrane integrity [17]. In this experiment, the primary hepatic cells were treated without or with different concentrations (1×10^{-5} , 10^{-6} and 10^{-7} M) of drug. After 24 h incubation, supernatant was removed from the hepatocytes and centrifuged to remove any cells/spheroids. Then LDH release from the hepatocytes was measured to evaluate the cytotoxicities of drug, compared with the control (without treatment of drug). The LDH assay was conducted using a commercially available kit from Nanjing Jiancheng Bioengineering Institute (China).

Acute Oral Toxicity – Fixed Dose Procedure. Fixed Dose Procedure was adopted to evaluate the acute oral toxicity of C according to Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals [18]. The female mice were selected for this experiment, because females are generally slightly more sensitive between the sexes [19]. Each animal, at the commencement of its dosing, is about 8 weeks old and its weight is fall in an interval within $\pm 20\%$ of the mean weight of any previously dosed mice. The Groups of 20 mice are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2000 mg/kg. The initial dose level is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. Clinical signs and conditions associated with pain, suffering, and impending death, are described in detail in a separate OECD Guidance Document [18]. Further groups of mice may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the dose causing evident toxicity or no more than one death is identified, or when no effects are seen at the highest dose or when deaths occur at the lowest dose.

Fig. 1. The structure of exifone (A), Chalcones (B), the polyphenol-3-methoxy-4, 2'-dihydroxy-5-bromo chalcone (C)

Spatial learning test with Morris Water Maze (MWM) test. All mice were grouped and treated as for tests of C on SOD, MDA and GSH-PX levels on scopolamine-induced Alzheimer's models (Table 4). We used a circular pool, 120 cm in diameter, with its inside painted dark black and filled up with tap water. Water was maintained at 22°C and the escape platform (diameter 15 cm), submerged 2 cm below the water surface, was placed at a certain position in the pool. Visual cues, such as colored shapes, were placed around the pool in plain sight of the animal. All animals underwent 2 tests per day from days 15 to 21 (days 1 to 7). For the hidden platform test, the mouse was released in the pool facing the wall at 1 of 4 points (north, south, east or west) and allowed to swim around the pool to search for the platform for 120 s. Mice that found or did not find the platform (but were guided to the platform) were allowed to stay on the platform for 30 s. The time taken to reach the platform (latency) was recorded. After drug administration on the morning of day 22, animals underwent a probe trial in which the platform was removed. Animals were placed in the pool at the same pole and allowed to swim for 120 s to search for the hidden platform, to confirm that the spatial navigation task was learned by use of environmental cues. The average swimming speed, the time spent in each quadrant of the pool and the times the mice swam across the "hidden" platform were recorded. After drug administration on the morning of day 23, animals underwent the visible platform test, in which the platform was made visible to eliminate any misinterpretation of results due to drug-induced impairment of vision. The latency finding the platform was recorded in this test.

Statistical Analysis

Data are shown as mean $\pm$ -SD, and analysis involved use of SPSS v10.0 (SPSS Inc., Chicago, IL) by one-way ANOVA followed by SNK-LSD test.

Results

Synthesis of C

C was successfully synthesized via Claisen-Schmidt condensation using pyridine as a catalyst. Spectral

Fig. 2. Concentration-clearance on DPPH[•] (A), Concentration-clearance on OH[•] (B)

Fig. 3. Global cerebral SOD, MDA, GSH-PX levels for all *, $P < 0.05$ vs blank group, **, $P < 0.01$ vs blank group; #, $P < 0.05$ vs model group; ##, $P < 0.01$ vs model group.

identifications of C are as follows: infrared (IR) spectroscopy (KBr, cm^{-1}): 3420-1, 1630-1, 1594-1, 1574-1, 1511-1 cm^{-1} ; $^1\text{H-NMR}$ (400 MHz, DMSO-d_6): 3.93 (s, 3H, OCH₃), 7.76 (d, $J=15.2$, 1H, CH=); 8.14 (d, $J=15.2$, 1H, CH=), 7.05~8.32 (m, 6H, ArH), 10.23 (s, 1H, OH), 12.76 (s, 1H, OH); mass spectroscopy (MS): 347.0 [M-H]⁺, 348.9 [M-2-H]⁺; EA: C: 46.12%; H: 3.04%.

Activity of C in scavenging DPPH and OH free radicals

Vitamin C, whose antioxidation effects was demonstrated in many experiments *in vitro* and in humans and must be ingested for nutrition, was selected as the

Fig. 4. Effects of C on LDH activity of the primary hepatocytes

positive control. After the test processes and statistical calculations, we obtained concentration-clearance (DPPH) curves (Fig. 2A). The IC_{50} DPPH were obtained for VC (0.241 mmol/L) and C (0.257 mmol/L) by SPSS, respectively. In the same way, concentration-clearance (OH) curve was obtained as follows (Fig. 2B). The IC_{50} OH were obtained for VC (0.442 mmol/L) and C (0.438 mmol/L). That the IC_{50} (DPPH) and (OH) values for C were slightly higher than that of VC indicated that C showed high activity as an antioxidant *in vitro*.

Impact of C on SOD, MDA and GSH-PX levels in scopolamine-induced AD model

In this study, we administrated scopolamine to stimulate AD [20, 21]. We measured SOD, MDA and GSH-PX levels to assess the potential of 3 levels of C (low, medium, high) against AD [22]. Piracetam, a classical AChE inhibitor for AD, was selected as a positive control rather than exifone, because exifone possessed acute toxicity and many former studies of AD involving similar models (scopolamine administration) adopted piracetam as a positive control [23, 24].

Fig. 3A shows the levels of SOD enzyme. The SOD level was significantly lower in model mice than in non-model mice ($p < 0.01$) and significantly higher in mice treated with piracetam or C than in model mice ($p < 0.01$). Fig. 3B shows levels of MDA. MDA level was significantly higher in model mice than in non-model mice ($p < 0.01$). MDA level were significantly lower with piracetam or C treatment than in model mice ($p < 0.01$). Fig. 3C shows levels of GSH-PX. GSH-PX level was significantly lower in model mice than in non-model mice ($p < 0.01$), and was significantly higher with piracetam or C treatment mice than in model mice ($p < 0.01$). From the effect of C on SOD, MDA and GSH-PX levels in scopolamine-induced AD model, it can come to a conclusion that C is a potential antioxidant *in vivo* in Alzheimer's models.

Toxic effects of C on mouse hepatocytes

Due to the hepatotoxicity of exifone, the evaluation of hepatotoxicity of C becomes necessary. In this experiment, LDH release from the primary hepatocytes was measured to evaluate the cytotoxicities of drug. The result is shown in Fig. 4. No significant differences in LDH release were observed for any treatment group after 24 h of drug exposure, compared with the control. Hence, no cytotoxicity was observed at least in these concentrations of drug.

Fig. 5. Latencies of each group in reaching the platform in spatial navigation test from days 1 to 7.

Fig. 6. The time each group spent in different quadrants. *, $P < 0.01$ vs blank group; #, $P < 0.05$ vs model group; ##, $P < 0.01$ vs model group.

Dose (mg/kg)	The result of the sighting study
5.0	100% survival rate, no severe toxic effects*
50.0	100% survival rate, no severe toxic effects*
300.0	100% survival rate, no severe toxic effects*
2000.0	100% survival rate, no severe toxic effects*

Table 5. The sighting study in acute oral toxicity assay—fixed dose procedure. *, Clinical signs and conditions associated with pain, suffering, and impending death, are described in detail in a separate OECD Guidance Document [19].

Group	day 1(s)	day 2(s)	day 3(s)	day 4(s)	day 5(s)	day 6(s)	day 7(s)
Blank	100.76±19.97	85.93±21.74	73.13±20.45	60.94±22.61	49.81±24.71	36.58±16.56	23.02±17.33
Model	110.27±23.82	109.66±20.68*	105.72±23.07*	102.36±26.91*	91.70±26.22*	83.19±23.79*	72.36±22.91*
Piracetam	104.67±20.16	92.36±20.90	77.43±22.09△△	64.90±24.67△△	53.86±18.05△△	40.11±12.25△△	29.99±11.56△△
C (low)	112.04±20.10	104.99±16.56	95.36±19.54	88.23±24.10▲	78.86±27.04▲	66.85±18.58▲▲	53.68±18.04▲▲
C (medium)	109.98±17.05	100.74±18.59	87.58±24.26	76.65±27.56△	69.19±21.43△	55.41±19.57△△	46.26±19.91△△
C (high)	105.16±24.95	95.61±24.13	80.07±22.53△	68.58±20.77△△	57.71±19.97△△	45.57±18.29△△	34.33±18.88△△

Table 6. Latencies of each group in reaching the platform in spatial navigation test from days 1 to 7 *, P < 0.01 vs blank group; △, P < 0.05 vs model group; △△, P < 0.01 vs model group; ▲, P < 0.05 vs piracetam group, P<0.05; ▲▲, P < 0.01 vs piracetam group.

Fig. 7. Times passing the hidden platform (A), Swimming speed of each was crossed by each group (B), Latencies of each group in visible platform test from days 1 to 7 (C). *, P < 0.01 vs blank group; #, P < 0.05 vs model group; ##, P < 0.01 vs. model group.

Acute oral toxicity of C on mouse

No mortality or severe toxic effects are seen even at the highest dose of 2000 mg/kg (Table 5). Hence, based on the criteria of OECD, C is of relatively low acute toxicity hazard and its expected LD50 values exceeds 2000 mg/kg without the need for testing.

Effect of C on spatial learning

We used the Morris water maze task (MWM) to test cognition and learning in mice because it has some advantages over conventional mazes such as the T-maze [25] (e.g., no local cues such as shape or scent and no fixed escape formula). After mice underwent the hidden platform water-maze test for 7 days, for all groups, the latency to reach the platform was reduced with increased days (Fig. 5 and Table 6). Scopolamine by intraperitoneal

injection successfully established the model of learning impairment with extended latencies from day 2 to day 7 in the model mice as compared with the non-model mice (p<0.01), and latencies were shorter with piracetam, C (high), C (medium) and C (low) groups than model treatment (p<0.05) from days 3, 3, 4 and 7, respectively. Therefore, piracetam and C at various doses could antagonize scopolamine-induced impaired learning to different degrees.

Probe trial test results showed that the piracetam and C treatment induced animals to spend more time than model animals in quadrant IV (the location of the platform) (p<0.01) and crossed the hidden platform more times (p<0.05) (Fig. 6 and 7A). Thus, animals learned to locate the platform by hints of special shapes around different quadrants, and scopolamine administration successfully

established the model of learning impairment ($p < 0.01$). Results in Fig. 7B and 7C showed that each group reached the visible platform at about the same time, so group differences in the spatial navigation test and probe trial test were not relevant to unidentified visible or kinematic injuries caused by drugs.

Discussion

The common route of synthesizing chalcones involves one step with Claisen-Schmidt condensation and hydroxyl-substituted benzaldehydes and acetophenones as synthesizing materials and with alkali (such as NaOH) as a catalyst at room temperature. However, hydroxyl substitutions on benzyl rings affected the reaction activity greatly, whereas other types of substitutions, including both electron-withdrawal and electron-donating groups, did not. Reports indicated that the reaction would fail with the total amount of hydroxyl-substitutions on benzaldehydes and acetophenones exceeding one [26]. The failure was probably due to hydroxy-substituted benzaldehydes as synthesizing materials, undergoing ionization in NaOH solution before the Claisen-Schmidt reaction started and converting into an ion form [27], and the carbonyl-C seemed to fail to keep its original polarity. However, acetophenones would provide an H to make the addition reaction proceed successfully. As well, a special problem existed during the process of adding material with hydroxyl-substituted benzaldehydes and acetophenones, that such benzaldehydes or acetophenones would undergo ionization in NaOH solution the instant they were added, so the mixture would be so thick as to not even be able to be stirred to complete the reaction. However, this problem could not easily be solved by adding more water as solvents because the concentration of alkali should be controlled in an adequate range: neither too low to catalyze the reaction nor too high to introduce unwanted byproducts of Cannizzaro reaction. Other reports exist of the synthesis of hydroxyl-substituted chalcones with acids used as catalysts or a hydroxyl protection and de-protection process following an alkali-catalysis when the total amount of hydroxyl-substituted benzaldehydes and acetophenones exceeded one [27, 28]. However, such shortcomings appeared as the process being too complicated or the yield too low. Pipyridine catalysis seems a highly effective and promising method in Claisen-Schmidt condensation synthesizing hydroxy-substituted chalcones.

A growing body of evidence suggests that AD

pathogenesis involves an imbalance between free radical formation and destruction. This concept was originally derived from the free radical hypothesis of aging, that age-related accumulation of free radicals results in damaged cell components. Tests of activities on DPPH and OH free radical scavenging served as classical methods assessing anti-oxidation properties *in vitro*. In this study, C showed high activity as an antioxidant *in vitro*. Additionally, from this oxidative-stress etiological theory of AD, we administrated scopolamine to stimulate AD. Then, we measured SOD, MDA and GSH-PX levels to assess the potential of 3 levels of C (low, medium, high) against AD [20-22]. Piracetam, a classical AchE inhibitor for AD, was selected as a positive control rather than exifone, because exifone was not available and many former studies of AD involving similar models (scopolamine administration) adopted piracetam as a positive control [23, 24]. The results indicate C possesses potential antioxidant activity *in vivo* in Alzheimer's models, according with the anti-oxidation properties *in vitro*.

In the study of MWM, results indicate that C was potent to some extent in antagonizing cognition and learning impairments induced by scopolamine stimulating Alzheimer's disease. In this experiment, we used mice, which are not considered natural swimmers. Some of the mice refused to stay on the platform even though they had already found it, which contributed to errors. MWM is a useful tool for anti-AD drugs screening [29]. MWM test mainly targets hippocampal spatial memory deficits [30]. And memory deficits in patients with AD present as declarative memory deficits, which is formed in reference spatial memory, and involves with limbic system. So MWM task to test cognition and learning in mice was used in the study of C's effect on spatial learning. It was reported that the effects of scopolamine were not only be related with spatial learning, but also related with age, pool wall brightness and pretraining [31-33] in a MWM task. So the age of mice, pool wall brightness and the number of pretraining were highly controlled in the same condition. Some studies reported systemic administration of scopolamine has widespread effects on physiological functioning including sensory processing, attention, learning and memory et al.. Its effects can be explained by the non-selective muscarinic binding characteristics of this ligand [33]. Furthermore, it was reported that systemic administration of scopolamine was more effective in disrupting acquisition than impairing retention in the Morris water maze [33, 34]. Even so, scopolamine can be used as a reference drug for inducing

cognitive deficits in healthy humans and animals, due to causing very potent performance impairment on tests of learning and memory [35].

In conclusion, C was confirmed to be highly potent in scavenging 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and OH free radicals *in vitro*. Tests of anti-free radical activity in response to oxidative stress in mice revealed that C could elevate glutathione peroxidase (GSH-PX) and super oxide dismutase (SOD) levels and lower malonaldehyde (MDA) level in a free-radical-injured scopolamine-induced Alzheimer's model. Further behavioral tests with the Morris water maze showed that C could antagonize the learning impairments in the Alzheimer's model, which suggests that C has a potential role in Alzheimer's disease.

In addition to its cognitive enhancing property, exifone has also been reported to cause severe hepatotoxicity, and then it has been withdrawn from the market [36].

Because C is similar to exifone in structure, eventual cytotoxicity of C should be evaluated. The test of acute oral toxicity indicated C is of relatively low acute toxicity hazard and its expected LD50 values exceed 2000 mg/kg. And no cytotoxicity was observed in the test of LDH Release.

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