

The chalcone derivative Chana 1 protects against amyloid β peptide-induced oxidative stress and cognitive impairment

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Abstract. Alzheimer's disease (AD) is the most common neurodegenerative disease to cause dementia in the elderly. Amyloid β (A β)-peptide induced oxidative stress causes the initiation and progression of AD. Recently, new chalcone derivatives termed the Chana series were synthesized. Among them, Chana 1 showed high free radical scavenging activity (72.5%), as measured by a DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. In this study, we investigated the effect of Chana 1 against A β -induced cytotoxicity and cognitive deficits. Additionally, we sought to estimate the lethal dose, 50% (LD₅₀) of Chana 1 in mice using an acute oral toxicity test. We found that Chana 1 significantly protected against A β -induced neuronal cell death in PC12 cells. Oral administration of Chana 1 at a dose of 50 mg/kg body weight/day significantly improved A β -induced learning and memory impairment in mice, as measured in Y-maze and passive avoidance tests. In acute toxicity tests, the LD₅₀ in mice was determined to be 520.44 mg/kg body weight. The data are valuable for future studies and suggest that Chana 1 has therapeutic potential for the management of neurodegenerative disease.

Introduction

Alzheimer's disease (AD) is the most frequent cause of dementia in the elderly and the number of AD cases continues to increase. AD is a progressive, irreversible neurodegenerative disease characterized by neurofibrillary tangles and senile plaques caused by deposits of amyloid β (A β) in the brain. A β protein and oxidative stress are believed to have crucial roles in the development of AD. A β is a source of free radicals itself in the presence of metal ions (1) or a pre-form of an aggregated A β that induces neurotoxicity by oxidative stress (2). The brain is particularly vulnerable to oxidative stress because of its elevated consumption of oxygen, high levels of polyunsaturated fatty acids, and relatively low levels of antioxidants (3,4). Several studies have observed increased protein oxidation (5) and reactive oxygen species (ROS) formation (6) in the brain tissue of AD patients. Moreover, A β is considered a cause of lipid peroxidation in brain cell membranes that may also contribute to neurodegeneration in AD brains (7,8). These data indicate that oxidative stress is an early event in AD pathogenesis.

In normal cellular metabolism, cells produce antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase as a part of a cellular defense system against ROS-mediated cellular damage (9). However, the endogenous antioxidant defense system becomes impaired with excessive generations of ROS and free radicals, such as is caused by increasing A β levels (10). These events make the brain vulnerable to A β -induced toxic effects and are involved in the pathogenesis of most of the neurodegenerative disorders, including AD. Multiple studies have shown that antioxidants and free radical scavengers inhibit A β -induced neurotoxicity (11-13) and antioxidant substances improve spatial cognition and retard AD pathogenesis (14,15).

Most polyphenols have strong antioxidant activity by reacting as hydrogen- or electron-donating agents, in addition to having metal ion chelating properties due to their phenol groups (16). Retrochalcone is an unusual phenolic compound that is structurally distinguished from normal chalcones by the

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lack of oxygen functionalities at C'-2 and C'-6. Recent studies have described the dynamic activities, including antioxidative and superoxide scavenging activity (17-21) of natural or synthesized retrochalcones.

In our previous study (22), we synthesized Chana 30, a retrochalcone derived via structural modification of a natural retrochalcone from licorice, and found the neuroprotective effect of Chana 30 was due to its potent free radical scavenging effect. In this study, we evaluated a new synthetic retrochalcone, Chana 1 that shows high antioxidant activity as compared with Chana 30. We investigated whether Chana 1 protects against neuronal cell death and cognitive impairment induced by A β peptide *in vitro* and *in vivo* and found a decrease in oxidative damage with improved cognition observed in mice. Additionally, we carried out an acute toxicity test to determine the oral lethal dose, 50% (LD₅₀) in mice. Our data suggest that Chana 1 is a possible chemotherapeutic agent for neurodegenerative disease.

Materials and methods

Materials. Fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid, and penicillin-streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich (St. Louis, MO, USA). A β ₁₋₄₂ and A β ₄₂₋₁ were purchased from Bachem (Bubendorf, Switzerland). All other chemicals were purchased from Sigma-Aldrich.

Cell culture. The rat pheochromocytoma PC12 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 media containing 10% heat-inactivated FBS and 1% penicillin-streptomycin. Cells were incubated at 37°C in a 5% CO₂ atmosphere with 95% humidity.

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. The free radical scavenging activity of Chana 1 was assayed using the DPPH method, a modification of the Blois method (23). Briefly, 10 mM of sample was dissolved in methanol and serially diluted to 5, 10, 20, 50 and 100 μ M with ethanol. DPPH (0.25 mM) was mixed with each diluted sample, incubated for 30 min at room temperature, and the optical density was measured at 517 nm. An equal volume of ethanol alone was added to the control tube. The DPPH radical scavenging activity was calculated according to the following equation: % scavenging activity = [(A₀-A₁)/A₀] x 100, where A₀ is the absorbance of control and A₁ is the absorbance of the sample.

Cell viability assay. An MTT assay was performed to test the cytotoxicity or neuroprotective effects of Chana 1 in PC12 cells. Briefly, cells (1x10⁴ cells/well) were seeded in 96-well culture plates and treated with various concentrations of Chana 1 for 24 h. Cell viability was expressed as a relative percentage against control cultures. For the measurement of A β -induced neuroprotective activity, various concentrations of Chana 1 were added to cells for 24 h before treatment with A β . The positive control drug, selegiline, currently being used to treat Alzheimer's, was evaluated in the same

manner for comparison. MTT solution (2.0 mg/ml) was added to each well and incubated for 4 h at 37°C. Media was removed and the formazan crystals obtained were solubilized in 100 μ l dimethyl sulfoxide (DMSO). The optical density was determined (excitation at 570 nm, emission at 630 nm) using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA).

Animals. ICR male mice (5-weeks-old) were obtained from Samtako Co. (Osan, Korea) and acclimated for 1 week prior to the initiation of the experiments. Animals were housed in a room maintained at a temperature of 23 \pm 1°C with 60 \pm 10% humidity and a controlled 12 h light/dark cycle. They were supplied with a standard pellet diet (Purina Korea) and tap water *ad libitum*. Mice were divided into 6 groups with 8 animals in each group: i) control group, ii) A β -injected group, iii) and iv) A β -injected with Chana 1 added, 20 or 50 mg/kg body weight, respectively, and v) and vi) A β -injected with selegiline (3.0 mg/kg body weight) and Aricept (1.0 mg/kg body weight), respectively. Chana 1 was dissolved in tap water and orally administered daily for 34 days. After administration of Chana 1 or control drugs for 28 days, A β ₁₋₄₂ or A β ₄₂₋₁ was administered by intracerebroventricular injection (410 pmol per mouse) according to Chauhan's method (24). The control group was injected with A β ₄₂₋₁, the non-toxic reverse fragment. A β peptides were dissolved in phosphate-buffered saline, and pre-incubated at 37°C for 5 days to allow fibril formation. The 10 μ l peptides were injected directly using a Hamilton microsyringe (25 G needle) into the third ventricle, 0.25 mm posterior to the bregma at a depth of 2.5 mm (anteroposterior, -0.25 mm; mediolateral, 0 mm; dorsal ventral, 2.5 mm relative to the bregma). All experiments were conducted in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of Yonsei University.

Y-maze test. A Y-maze was used to test for ameliorating effects of Chana 1 in mice on spatial working memory 4 days after A β injection. The used maze was 33 cm long, 15 cm high, and 10 cm wide with the arms symmetrically disposed. Each mouse was initially placed within one arm and number of arm entries was recorded manually during an 8-min period. The percentage of alternation was determined using the following equation: % alternation = [(number of alternations)/(total arm entries-2)] x 100 (25).

Passive avoidance test. A passive avoidance test was used to evaluate the effects of the Chana 1 on learning and memory (26). The test was carried out 6 days after A β injection. A two-compartment step-through passive avoidance apparatus (Model PACS-30; Columbus Instruments International, Columbus, OH, USA) was used. The apparatus was divided into two chambers of equal size (23.5x15.5x15.5 cm), one illuminated and one dark. During the training session, mice were placed in the illuminated compartment and the door was raised to allow the mice to enter the dark compartment. When the mice entered the dark compartment, the door was closed and an electrical foot shock (0.5 mA for 1 sec) was delivered. Control mice were also subjected to electric foot shock when entering the dark compartment. The testing session was performed 1 day after the training session. The mice were

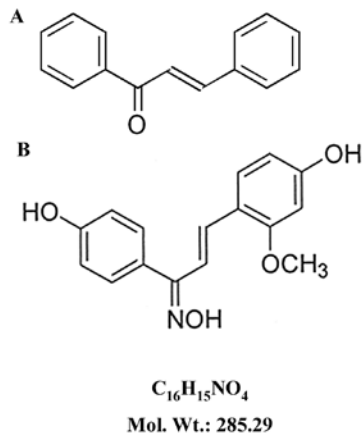


Figure 1. Chemical structures of chalcones. (A) Natural chalcone backbone and (B) synthesized Chana 1.

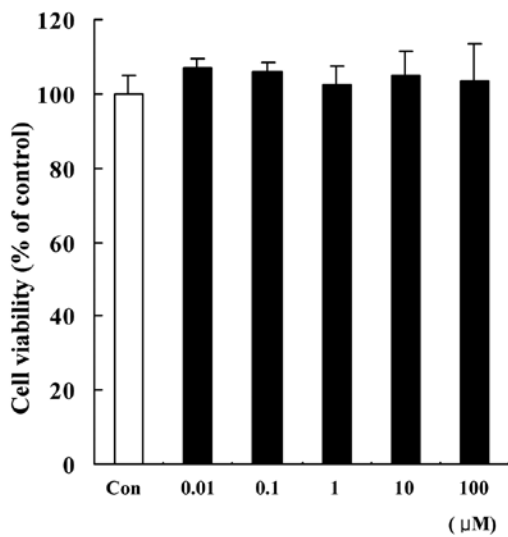


Figure 2. Effects of Chana 1 in the PC12 cell viability assay. PC12 cells were treated with Chana 1 of various concentrations (0.01-100 µM) in serum-free media for 24 h. The cell viability was determined by the MTT assay, and expressed as the mean percentage of viable cells compared with the untreated cells. The data are the mean ± SE (n=5).

again placed in the bright compartment and the latency time to enter the dark compartment was recorded (up to 300 sec).

Evaluation of acute oral toxicity. Acute oral toxicity (LD₅₀) was evaluated by a mouse lethality assay. The acute toxicity test was performed according to the Organisation for Economic Co-operation and Development guidelines (27). Mice were randomly assigned to 5 groups of 10 animals each. The control group received a mixture of saline and 0.5% carboxymethyl cellulose (CMC) only. A mixture of saline and 0.5% CMC with 5% DMSO was used for the preparation of the different doses of Chana 1 (300, 500 and 700 mg/kg body weight), that was used as a vehicle. All treatment was given by gavage for oral administration. The mice were observed 1 h after a single administration of Chana 1 and subsequently monitored until 96 h post treatment. Acute toxicity was calculated as the dose required (in mg/kg body weight) to cause death in 50% of animals (LD₅₀) using a Sigma plot program.

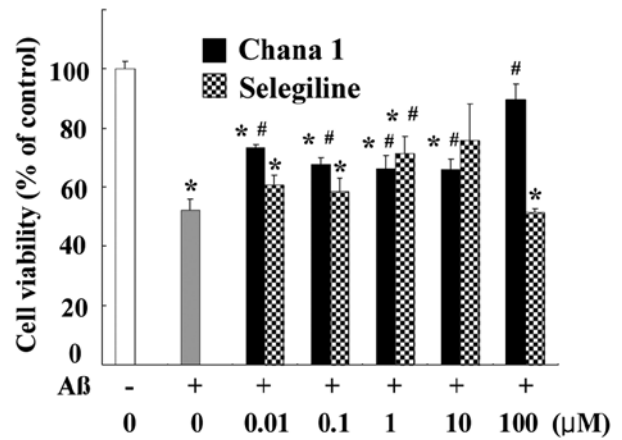


Figure 3. Protective effects of the Chana 1 against Aβ₁₋₄₂-induced cell damage. Cell viability of Chana 1 was evaluated using the MTT assay. All groups were treated with 25 µM Aβ₁₋₄₂ except for the control group. Selegiline was used as a positive control at the same concentrations. Chana 1 and selegiline were pre-incubated at various concentrations (0.01-100 µM) in serum-free media for 24 h before the addition of Aβ peptide. Cell viability is expressed as the mean percentage of viable cells compared with the untreated cells. The data are the mean ± SE (n=5). *P<0.05 vs. the control group; #P<0.05 vs. the Aβ₁₋₄₂-peptide treated group.

Statistical analyses. All results are expressed as means ± standard error. Statistical comparisons were performed using a Student's t-test. A P-value <0.05 was considered to indicate statistical significance.

Results

Cytotoxicity of Chana 1 in PC12 cells. In our previous study, we screened the synthetic chalcone series using the free radical scavenging assay. This assay is considered a preliminary screening tool to determine whether compounds have scavenging activity against oxygen species (28). In this study, Chana 1 was found to have potent DPPH radical scavenging activity with an activity of 76.5%. The chemical structure of synthetic Chana 1 is shown in Fig. 1B. Next, we conducted an MTT assay to evaluate the effect of Chana 1 on cell viability. PC12 cells were incubated with varying concentrations (0.01-100 µM) of Chana 1 for 24 h. Chana 1 did not exhibit cytotoxicity in PC12 cells at any of the concentrations tested (Fig. 2).

Protective effect of Chana 1 against Aβ-induced cell cytotoxicity in PC12 cells. To evaluate the effect of Chana 1 on Aβ-induced PC12 cell death, we also performed MTT assays in cells treated with varying concentrations of Chana 1. Cells treated with Aβ demonstrated significantly decreased cell viability as compared with control cells (Fig. 3). However, pretreating the cells with Chana 1 significantly protected against Aβ-induced cell death (up to 89.57%) at a concentration of 100 µM. Moreover, the protective effect of Chana 1 was significantly higher than that of selegiline. These results suggest that Chana 1 can protect against the cell damage caused by Aβ-induced cytotoxicity.

Ameliorative effects against Aβ-induced memory impairment of Chana 1 in mice. Given the protective activity of Chana 1

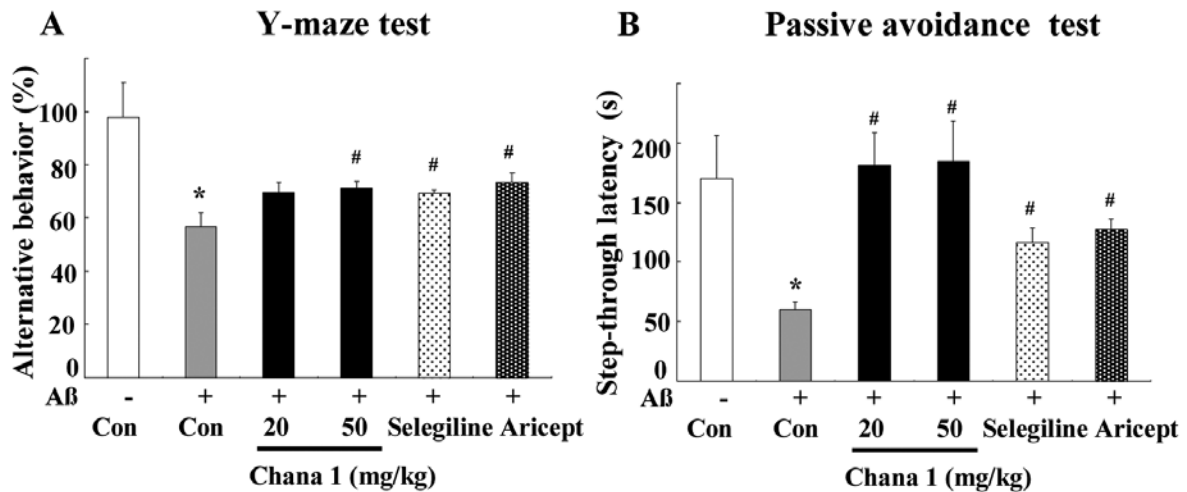


Figure 4. Effects of the Chana 1 against A β -induced memory impairment in mice. (A) Y-maze test was carried out for spontaneous alternation behavior, which is regarded as a measurement of spatial memory in A β -injected mice. (B) Step-through latency (s) was evaluated for a passive avoidance task in A β -injected memory-impairment mice. Chana 1 (20 or 50 mg/kg b.w.), selegiline (3 mg/kg b.w.) or Aricept (1 mg/kg b.w.) were given by oral administration for 28 days before A β ₁₋₄₂-injection; control mice were injected with A β ₄₂₋₁. The data are the mean \pm SE (n=8). *P<0.05 vs. A β ₄₂₋₁-injected control group; #P<0.05 vs. A β ₁₋₄₂-injected group.

Table I. Effects of oral administration of Chana 1 on body weight (b.w.) through at the experiment.

Experimental group	Initial b.w. (g)	Final b.w. (g)
Control	20.3 \pm 1.3	35.8 \pm 0.6
A β ₁₋₄₂	20.3 \pm 1.4	36.7 \pm 0.6
A β ₁₋₄₂ + Chana 1 (20 mg/kg b.w.)	21.0 \pm 1.4	35.5 \pm 0.6
A β ₁₋₄₂ + Chana 1 (50 mg/kg b.w.)	19.3 \pm 1.4	36.7 \pm 1.0
A β ₁₋₄₂ + selegiline (3 mg/kg b.w.)	19.8 \pm 1.4	37.0 \pm 0.7
A β ₁₋₄₂ + Aricept (1 mg/kg b.w.)	20.0 \pm 1.4	36.0 \pm 0.7

Control mice were injected with A β ₄₂₋₁. The data are the mean \pm SE (n=8).

Table II. Effects of Chana 1 in mice after acute oral administration.

Treatment dose (mg/kg)	Mice		Effects	
	Gender	D/T	Mortality latency (h)	Symptoms of toxicity
Control	M	0/5	-	None
	F	0/5	-	None
Vehicle	M	0/5	-	None
	F	0/5	-	None
300	M	1/5	>6, <24	Slow movement, eye mucus
	F	3/5	>24, <27	Lying down, eye mucus
500	M	4/5	>6, <24	Slow movement, hair bristling
	F	1/5	>27, <31	Slow movement, hair bristling
700	M	4/5	>6, <24	Lying down, eye mucus, hair bristling
	F	2/5	>6, <24	Lying down, eye mucus, hair bristling

M, male; F, female; D/T, dead/total treated mice; mortality latency, time to death after the oral injection.

in vitro, we performed an *in vivo* study to investigate the effects of Chana 1 on the learning and memory impairment in A β -injected mice. We performed a Y-maze test to evaluate Chana 1 on the spatial working memory of mice. Spatial working memory is a form of short-term memory recognition measured by recording spontaneous alternation behavior. Selegiline and Aricept, are being used to treat Alzheimer's, were used as positive controls. We found that the spontaneous alternation behavior of the A β ₁₋₄₂-injected group was significantly lower than that of the control groups ($P < 0.05$) (Fig. 4A). However, the reduced spatial working memory by A β ₁₋₄₂ treatment was significantly ameliorated by the administration of 20 and 50 mg/kg Chana 1 (12.9 and 14.2%, respectively). There were no differences in the positive controls upon addition of Chana 1. In addition, we evaluated learning ability by measuring the response latency in the step-through passive avoidance test. The behavior of the A β ₁₋₄₂-injected group indicated significant damage as measured by the passive avoidance test. The step through latency (STL) of the control group and A β ₁₋₄₂-injected group were 170.3 and 60.0 sec, respectively. The Chana 1 treated group dramatically improved A β -induced memory impairment as measured by an increase in STL time (Fig. 4B). Moreover, these results were significantly higher than the positive controls selegiline (116 sec) and Aricept (127 sec; $P < 0.05$). These results demonstrate that Chana 1 can improve A β -induced learning and memory impairment in mice. During the study, all group of mice gained weight regularly (Table I).

Determination of the LD₅₀ of Chana 1 in mice. It is critical that an active compound has its safety assessed to be valuable for its biological efficacy. Thus, an oral acute toxicity test of Chana 1 was carried out. The LD₅₀ of Chana 1 in mice was determined after a single oral administration. The behavior of the mice and number of deaths were observed for 96 h. The effects of oral administration of a single dose of Chana 1 are summarized in Table II. We found a dose-dependent increase in mortality and adverse effects. The mortality rate increased to 60% at the highest dose tested (700 mg/kg) and males were more sensitive than females. We determined the latency of mortality and observed it up to 31 h. The symptoms of toxicity, such as slow movement, hair bristling, lying down, and eye mucus were similar in both genders. From these data, we estimate the oral LD₅₀ of Chana 1 in mice to be 520.44 mg/kg body weight.

Discussion

In the present study, we determined that the chalcone derivative, Chana 1, has neuroprotective effects against A β -induced oxidative stress and memory impairment. Oxidative stress has consistently been implicated in the neuronal cell death and dysfunction observed in chronic neurodegenerative diseases, including Alzheimer's disease (AD) (29,30). Furthermore, it is well known that the A β peptide generates ROS directly inside cell membranes to cause protein oxidation and lipid peroxidation (31) as well as neuronal cell death (32). Oxidative damage is clear in the postmortem examination of brains from patients with AD due to the presence of oxidative DNA, lipid peroxidation, modification of proteins, and other indicators of ROS damage such as 4-hydroxy-2-trans-nonenal and malondialdehyde (33). Given the involvement of ROS in the pathogenesis

of AD, free radical scavengers and antioxidants have been suggested as possible therapies. In addition, many studies have reported that antioxidant activity reduces the neuronal cell death and memory impairment caused by A β peptide (34-37).

Currently, there are two types of drugs on the market for AD: acetylcholinesterase inhibitors (e.g., Aricept, Tacrine) and N-methyl-D-aspartic acid receptor antagonists (e.g., Memantine). However, these drugs reduce the symptoms of the disease, rather than truly modifying the disease (38). Also, these drugs have negative side effects including nausea, vomiting, insomnia, orthostatic hypotension, and micturition dysfunction (39). Therefore, the search continues for more effective and less toxic therapies.

In a previous study, we determined that licochalcone derivatives, the Chana series, showed potential free radical scavenging activity. Chana 1 was one of the most potent free radical scavengers and we hypothesized it would be effective against A β -induced neuronal damage.

Here, we demonstrated that Chana 1 has the ability to reduce A β -induced neurotoxicity in PC12 cells. Treating mice with 20 or 50 mg/kg Chana 1 also ameliorated the impaired learning and memory as much as the positive control drugs. The antioxidant capacity of Chana 1 appears responsible for the neuroprotective effect due to the attenuation of oxidative stress. Moreover, the toxicity of Chana 1 was less than either selegiline or Aricept as measured by an acute toxicity test in mice. The LD₅₀ value of Chana 1 was determined to be 520.44 mg/kg body weight, whereas the LD₅₀ of selegiline was 385 mg/kg body weight (in the rat) (40) and the LD₅₀ of Aricept was 45.2 mg/kg body weight (in mice) (41). According to the Hodge and Sterner scale (42), an LD₅₀ within the range of 500-5000 mg/kg is considered slightly toxic (class 4), 50-500 mg/kg is considered moderately toxic (class 3), and 1-50 mg/kg is highly toxic (class 2). Thus, according to this scale, Chana 1 is considered less toxic than either selegiline or Aricept. Although chalcone derivatives receive attention due to their wide variety of pharmacological activities, there is no information on their safety or acute toxicity *in vivo*. Therefore, our acute toxicity study provides data for future clinical studies and information for the safe usage of chalcone derivatives.

In conclusion, we report that Chana 1 protects against A β -induced oxidative stress and improves learning and memory impairment after A β injection in mice. We suggest that the free radical scavenging activity of Chana 1 is responsible for its neuroprotective effects. Our results indicate that Chana 1 may be a promising agent to slow the progression of AD. Additionally, this study provides data on the acute toxicity of Chana 1 that could be useful for future studies.

Acknowledgements

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