Effects of *Gentiana delavayi* Flower Extract on APP Processing in APP/PS1 CHO Cells

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Received August 26, 2019; accepted February 20, 2020

Ethnopharmacological relevance: *Gentiana delavayi* Franch. (Gentianaceae) as an ethnomedicinal plant contains a variety of effective active ingredients and exhibits diverse pharmacological actions, such as hepatoprotective, anti-inflammatory and central nervous system effects. In this study we investigated the influence of *G. delavayi* flower extract on amyloid precursor protein (APP) processing at molecular and cellular levels. APP/PS1 Chinese hamster ovary (CHO) cells were treated with chloroform extract of *G. delavayi* flower in different concentrations for 24 h. Concentrations of amyloid β (Aβ) 40 and Aβ42 in the cell supernatant and activity of β-site amyloid precursor protein cleaving enzyme 1 (BACE1), BACE2, and cathepsin D were determined. The expression of APP and neprilysin (NEP) within the cell were further determined. Compared with the control group, the levels of Aβ40 and Aβ42 declined notably and the activity of BACE1 was inhibited significantly in the APP/PS1 CHO cells after treatment with the chloroform extract of *G. delavayi* flower. Although the activities of BACE2 and cathepsin D were not changed, the expression of Aβ degrading enzyme NEP increased remarkably. Our experiments have clearly showed that the chloroform extract of *G. delavayi* flower inhibits the generation of β-amyloid by specifically inhibiting β-secretase and increases the expression of NEP which fastens the degradation of Aβ, exhibiting the effect of decreasing Aβ accumulation in APP/PS1 CHO cells. These results suggest that the active components from the chloroform extract of *G. delavayi* flower have a further prospect to be developed as potential anti-Aβ drug.

**Key words** Alzheimer’s disease; *Gentiana delavayi* extract; ethnomedicinal plant; amyloid precursor protein; cathepsin D; neprilysin

INTRODUCTION

Alzheimer’s disease (AD) is the primary dementia-causing disease and approximately 50 million people worldwide have dementia in 2019.1 As the disease advances, symptoms include disorientation, deepening confusion about events, loss of motivation, failure to perform self-care, and finally the loss of bodily functions leading to death. The pathological changes of AD are large deposition of amyloid β (Aβ) peptides and intracellular neurofibrillary tangles.2 Aβ are generated by the lysis of amyloid precursor protein (APP). APP is proteolytically processed by α-, β-, and γ-secretases. The three proteases process APP in two different pathways, one of which is called amyloidogenic and another is anti-amyloidogenic. In the amyloidogenic pathway APP is first cleaved by β-site amyloid precursor protein cleaving enzyme 1 (BACE1)3 and generates soluble β fragment of amyloid precursor protein (sAPPβ) and C-terminal fragment β (CTF-β). γ-Secretase further cleaves CTF-β to release APP intracellular domain (AICD) and Aβ, which aggregates to form amyloid plaques.4 Among all Aβ isoforms, Aβ40 and Aβ42 are believed to be the most important ones.5 In recent years, several studies have demonstrated that Aβ42 could form oligomers significantly faster than Aβ40, and the oligomers show the greatest level of neurotoxicity.5,6 The more generally accepted hypothesis of the pathogenesis about AD indicates that the effect of Aβ oligomers on neurons and astrocyte plays an important role in the development of AD.8,9 In the discovery of inhibitors of Aβ production, BACE1, as a rate-limiting enzyme for Aβ generation, has received much attention.10 However, no BACE1 inhibitor has passed clinical trials11. But the activity of BACE1 was still suggested as a new approach for anti-AD drug discovery12 based on the fact that suppression of BACE1 activity efficiently improves AD symptoms.12 Beside the production of Aβ by the amyloidogenic processing of APP and β-secretase, Aβ levels are also determined by its degradation, mediated mainly by neprilysin (NEP).13 All of the currently available cholinesterase inhibitors (ChEIs: donepezil, galantamine, and rivastigmine) are indicated for mild-to-moderate AD. They remain to be symptomatic drugs with no success in preventing, treating or curing the disease.14 Advances in research and development of new drugs for the prevention and treatment of AD is not very fortunate, and a very high attrition rate was found, with an overall success rate during the 2002 to 2012 period by 0.4%.15 Therefore, a multi-target-based strategy has gradually been accepted as a promising trend for the discovery of drug compounds against AD.16 Natural products have been recognized as sources of new lead compounds for the treatment of various diseases, including AD.17 Luckily, an extract of *Gentiana delavayi* flower was discovered by us to inhibit BACE1 activity and increase the expression of NEP.

*Gentiana delavayi* Franch. (Wei Zi Long Dan by Chinese name) grows in southwest of China and is a well-known traditional but pharmacologically less explored Chinese herbal medicine.18 It is an ethnomedicinal plant used for choleretic,
antihepatotoxic, antioxidative, and anti-inflammatory purposes. However, little is known about its neuroactive potential on AD. In a latest study performed in our laboratory, we investigated the inhibitory effects of chloroform extract of *G. delavayi* flower on acetyl cholinesterases (AChE). Our results showed that the chloroform extract of *G. delavayi* flower inhibits AChE activity significantly. And its inhibiting rate at 100 µg/mL was 51.1% by comparison with huperine A at 500 nM was 92.4%.  

It seems only that inhibiting the cholinesterase is not ideal for the development of AD drugs. However, previous studies showed that the ethnomedicinal plant was used as anti-inflammatory, and antioxidative agent. We speculate that the components within the extract may show some other positive effects. Then it will show the property of multi-target action and have a better potential for further research and development. Therefore, in the present study, we examined the effect of chloroform extract of *G. delavayi* flower on the Aβ40 and Aβ42 secreted in APP/PS1 Chinese hamster ovary (CHO) cells. We also analyzed whether the extract change the intracellular APP, and the activity on BACE1 *in vitro* and NEP protein levels.

**MATERIALS AND METHODS**

*G. delavayi* **Plant Collection** Gentiana delavayi Franch. (Gentianaceae) source plant material was collected in October 2013 from Er Yuan, Yunnan Province, China. The plant was identified and authenticated by Dr. Baozhong Duan, a plant taxonomist at Dali University, Yunnan, China.

*G. delavayi** **Characterization and Extract Preparation**  
*G. delavayi* source plant material was identified. A herbarium voucher specimen has been kept at our research group (W201310). An extract from dried ground *G. delavayi* flowers was prepared at Dali University.

The plant material was dried in room temperature and powdered. Three and half kilograms of the dry flower powder was extracted with 12L of 95% (v/v) ethanol/water for 3 times (12h each) in simply stirring the powder at room temperature. The extractives were combined, concentrated under reduced pressure and dried in vacuum to remove the solvent. The crude extract (687 g) was suspended in 5L distilled water, the extracted with 3L of chloroform for 3 times. The chloroform extracting solution was also concentrated until dry to provide *G. delavayi* chloroform extract (143 g). The chloroform extract (143 g) was subjected to silica gel column chromatography (CC) eluted with CHCl3/MeOH gradient (50:1 to 5:1) to afford seven fractions (A–G) on the basis of TLC analyses. Fr. D (13 g) was subjected to Sephadex LH-20 CC to give two fractions (Fr. D1 and Fr. D2). Fr. D1 was further separated by CHCl3/MeOH gradient (40:1 to 30:1) and the yield was named the chloroform extract of *G. delavayi*. The yield was 4.75% (w/w) and was stored at −20°C. Further phytochemical investigation of the chloroform extract led to the isolation of two known compounds (+) syringaresinol-4-O-β-D-glucopyranosid (1), apigenin (2) and a new compound named 7,2,3′,6′-tetraacetyl-4′-O-(2-hydroxy-3-O-β-D-glucopyranosyl-benzoyl)loganin (3). These structures were elucidated by spectroscopic methods including 1D NMR, 2D-NMR, and high resolution (HR) MS. Standard compounds 1–3 were finally purified by semi-PHPLC, and the purities of the compounds were more than 98%.

**HPLC Analysis of the Chloroform Extract of *G. delavayi*** The chloroform extract of *G. delavayi* was dissolved in methanol. That solution was filtered using a Greenherbs NYLON 13 mm syringe filter (0.45 µm, Beijing, China), and then filtrates were subjected to HPLC. The analytical column was a Agilent Eclipse XDB-C18 (Agilent Technologies, Santa Clara, CA, U.S.A.) and was kept at 30°C during performance. The data were analyzed by Chemstation software (Agilent Technologies). The mobile phase conditions contained solution A 0.1% phosphoric acid in water and acetonitrile. The gradient flow was as follows; 0–8 min, 5–14% D1; 8–13 min, 14–20% D1; 13–27 min, 20–25% D1; 27–52 min, 25–36% D1; 52–73 min, 36–42% D1; 73–75 min, 42–66% D1; 75–90 min, 66–100%. The injection volume was 10 µL, and the analysis was carried out at a flow rate of 1.0 mL/min with detection at 254 nm.

**Cells and Cell Culture Conditions** APP/PS1 CHO cells were maintained in Dulbecco’s modified eagle medium (DMEM) (Thermo Fisher Scientific, U.S.A.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 U penicillin–0.1 mg/mL streptomycin (Thermo Fisher Scientific) at 37°C with 5% CO2 in a tissue culture incubator (Thermo Fisher Scientific). The cells were passaged every 2 d when they reached 80% confluence.

**Cell Viability Assay** To explore the cytotoxic effect of *G. delavayi* chloroform extract on APP/PS1 CHO cells, cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Kit (JianCheng, China). The cells were treated with increasing doses of the chloroform extract of *G. delavayi* (0.05, 0.5, 5, 50, 500 µg/L) for 24h. The MTT assay was performed after the treatments. The cells were incubated for 4h at 37°C with 0.5 mg/mL of MTT dissolved in fresh complete medium. The extract was dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, U.S.A.), and the absorbance was measured on a microplate reader using a test wavelength of 570 nm. The data were expressed as the mean percentages of viable cells versus the control.

**Aβ, BACE1, BACE2 and Cathepsin D Enzyme-Linked Immunosorbent Assay (ELISA)** The cells were incubated with the chloroform extract of *G. delavayi* (0.05–500 µg/L) for 24h. After treatment, conditioned media from the treated and untreated cells were collected to detect secreted Aβ40, Aβ42, BACE2 and cathepsin D. The Aβ40 (Xinlebio, Shanghai), Aβ42 (Xinlebio), BACE1 (Raybiotech, China), BACE2 (Mybiosourse, U.S.A.) and cathepsin D (Abcam, U.K.) concentrations were quantified using ELISA kits following the manufacturer’s protocol. The optical densities of each well at 450 nm were read on a microplate reader, and the sample Aβ40, Aβ42, BACE1, BACE2 and cathepsin D concentrations were determined by comparison with the standard curves. All readings were in the linear range of the assay.

**Western Blotting Analysis of APP and NEP** After treatment, the cells were washed with ice-cold phosphate buffer saline (PBS, pH = 7.4) and then lysed in Radio Immunoprecipitation Assay (RIPA) lysis buffer containing cocktail of complete protease inhibitors. The protein concentrations were determined by bicinechonic acid (BCA) method (Beyotime, China). Protein from the treated cells was mixed with loading buffer, boiled for 5 min. Then, the protein (15 µg) was loaded.
and separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk, the membranes were subsequently incubated with primary antibodies against APP (1:1000) (Signalway Antibody, U.S.A.), NEP (1:1000) (ABclonal, China) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000) (Bioworld, U.S.A.) in Tris-buffered saline Tween-20 (TBST) containing 5% bovine serum albumin (BSA) overnight and washed three times for 10 min in TBST with gentle agitation. These membranes were incubated with the corresponding anti-mouse or anti-rabbit immunoglobulin G (IgG) (Cell Signaling Technology, U.S.A.) peroxidase secondary antibodies in TBST containing 5% BSA at room temperature for 1 h. After washing, the bands were detected using an enhanced chemiluminescent (ECL) kit.

**Statistical Analysis** The data were expressed as the mean ± standard deviation (S.D.) of cell treatments from at least three independent experiments. Repeated measures of ANOVA have been applied to Figs. 3 and 4. Turkey’s analysis had been used for the comparison with each group. For Figs. 2 and 5, one-way ANOVA with Turkey’s analysis was carried out. p-Value of less than 0.05 was significant. The statistical software GraphPad Prism 5, version 5.03, was used for all data analyses.

**RESULTS**

**Compounds Isolated from the Chloroform Extract of G. delavayi** The chloroform extract of G. delavayi flower was subjected to HPLC analysis (Fig. 1A). The compounds isolated from the extract was subjected to HPLC analysis. Compounds 1, 2, and 3 were detected at retention times of 26.9, 51.7 and 62.9 min, and the concentration of compounds 1, 2, and 3 following quantitative analysis were 4.0 ± 0.0, 34.6 ± 0.1 and 75.2 ± 0.0 µg/mg, respectively. The compound 3 was a new compound.

Structural identification of compounds 1–3 (Fig. 1B). Compound 1 was obtained as yellow powders. $^1$H-NMR (400 MHz, CDCl$_3$) δ: 3.08 (m, 2H, H-1,5), 4.73 (t, J = 4.3 Hz, 2H, H-2,6), 6.57 (s, 2H, H-2'/uni2033,6'/uni2033), 6.58 (d, J = 3.0 Hz, 2H, H-2'/uni2032,6'/uni2032), 4.58 (d, J = 7.6 Hz, 1H, H-glc-1), 3.86 (s, 6H, 2 × -OCH$_3$), 3.89 (s, 6H, 2 × -OCH$_3$); $^{13}$C-NMR (100 MHz, CDCl$_3$) δ: 54.4 (C-1), 85.9 (C-2), 72.0 (C-4), 54.6 (C-5), 86.2 (C-6), 72.1 (C-7), 132.1 (C-1'), 102.9 (C-2',6'), 147.3 (C-3',5'), 134.7 (C-4'), 138.8 (C-1''), 103.0 (C-2'',6''), 153.0 (C-3'',5''), 134.5 (C-4''), 106.2 (C-glc-1), 153.0 (C-3'',5'').

**Fig. 1.** Compounds Isolated from the Chloroform Extract of G. delavayi
(A) HPLC chromatograms of the chloroform extract of G. delavayi. (B) Structures of compounds 1–3. (C) COSY (bold lines) and key HMBC (1H–$^{13}$C) (arrows) correlations of 3.
Compound 2 was obtained as yellow powders. \(^1H\)-NMR (400 MHz, DMSO-d\(_6\)) \(\delta\): 6.8 (s, 1H, H-3), 6.2 (d, \(J = 1.9\) Hz, 1H, H-6), 6.54 (d, \(J = 1.9\) Hz, 1H, H-8), 7.91 (d, \(J = 8.8\) Hz, 2H, H-2',4'), 6.96 (d, \(J = 8.8\) Hz, 2H, H-3',5'); \(^{13}C\)-NMR spectrum (100 MHz, DMSO) \(\delta\): 164.4 (C-2), 102.8 (C-3), 181.7 (C-4), 157.3 (C-5), 94.0 (C-6), 163.8 (C-7), 98.9 (C-8), 161.4 (C-9), 103.6 (C-10), 121.0 (C-1'), 128.4 (C-2'), 116.0 (C-3'), 161.3 (C-4'), 116.0 (C-5'), 128.4 (C-6'). It was determined as apigenin by comparison with the literature.\(^{29}\)

Consistent with these observations, a 2,3-dihydroxybenzoyl moiety of the loganin unit in compound 3 was connected to C-1 by an ether linkage. This conclusion was also confirmed by the correlation from H-7 (\(\delta_H 5.14\)) to H-3' (5.51) and H-6' (4.25, 4.30) to four carbonyl carbons (\(\delta_C 170.8, 171.4, 172.2, 172.6\)). The nuclear Overhauser effect spectroscopy (NOESY) correlations revealed that H-5, H-9, and H-10 were \(\beta\)-oriented, whereas, H-1, H-7, and H-8 were \(\alpha\)-oriented. Consequently, the structure of compound 3 was unambiguously identified as \(7,2',3',6'\)-tetraacetyl-4'-O-(2-hydroxy-3-\(\beta\)-O-glycosyrylosybenzoyl)loganin.

**Effect of the Chloroform Extract of G. delavayi on Cell Viability of APP/PS1 CHO Cells**

APP/PS1 CHO cells were treated with a dose range of the chloroform extract of *G. delavayi* (0.05–500 \(\mu\)g/L) for 24 h as described in Materials and Methods, the MTT result showed that compared to the control group, the chloroform extract of *G. delavayi* appeared not to alter cell viability. This result indicates that the chloroform extract of *G. delavayi* treatment within the range of 0.05–500 \(\mu\)g/L for 24 h is safe for APP/PS1 CHO cells.

**The Chloroform Extract of G. delavayi Attenuates A\(\beta\)40 and A\(\beta\)42 Secretion from the APP/PS1 CHO Cells**

After chloroform extract of *G. delavayi* treatment, as shown in Figs. 2A and B, chloroform extract of *G. delavayi* at range from 0 to 500 \(\mu\)g/L significantly decreased the extracellular levels of A\(\beta\). The exposure of APP/PS1 CHO cells to *G. delavayi* for 24 h resulted in a significant decrease of A\(\beta\), in which A\(\beta\)40 was reduced to 31.5 ± 6.12% of control (\(p<0.01\)) and A\(\beta\)42 to 27.3 ± 8.04% of control (\(p<0.01\)).

**The Chloroform Extract of G. delavayi Suppresses the Activity of Secreted BACE1 in the Conditioned Medium of APP/PS1 CHO Cells**

As shown in Fig. 3, ELISA Kit analysis revealed lower activities of BACE1 in APP/PS1 CHO cells supernatant, in comparison with the control group and the positive group. The chloroform extract of *G. delavayi* and

| Position | \(\delta_C\) | \(\delta_H\) | Position | \(\delta_C\) | \(\delta_H\) |
|----------|-------------|-------------|----------|-------------|-------------|
| 1        | 96.9, CH    | 5.22 (d, 3.3) | 3'       | 147.3, C    | 7.48 (dd, 8.0, 1.5) |
| 3        | 151.2, CH   | 7.26 (d, 1.1) | 4'       | 124.8, CH   | 6.87 (t, 8.0) |
| 4        | 115.3, C    | 5'          | 5'       | 120.3, CH   | 7.44 (dd, 8.0, 1.5) |
| 5        | 31.5, CH    | 2.97 m      | 6'       | 124.1, CH   | 8.12% of control (\(p<0.01\)) and A\(\beta\)42 to 27.3 ± 8.04% of control (\(p<0.01\)).

**Table 1.** \(^1H\)-NMR and \(^{13}C\)-NMR Spectroscopic Data for Compound 3 (\(\delta\) in ppm, \(J\) in Hz) in Methanol-d\(_4\)
AZD3293, a BACE1 inhibitor (Astra-Zeneca), pretreatment for 24 h significantly attenuated the activity of BACE1 dose-dependently in APP/PS1 CHO cells supernatant. And maximal inhibitory efficiency at 500 µg/L for 24 h was 55.2 ± 0.6 and 64.5 ± 1.85%, respectively. (p < 0.01).

The Chloroform Extract of G. delavayi Does Not Inhibit BACE2 and Cathepsin D As shown in Fig. 4, the extract does not bind to BACE2 (data not shown) or cathepsin D (Fig. 4), the highly related protease to BACE1. Together, these data indicate that the extract is a potent and exquisitely selective BACE1 inhibitor.

The Chloroform Extract of G. delavayi Increased NEP Proteins in APP/PS1 Cells We further investigated whether chloroform extract of G. delavayi could regulate the expression of APP and NEP proteins in APP/PS1 CHO cells. As shown in Fig. 5, no changes of APP protein (data not shown) were found in APP/PS1 CHO cells with or without chloroform extract of G. delavayi treatment, the protein level of NEP in APP/PS1 CHO cells was higher than that of control group.

DISCUSSION

The amyloid cascade hypothesis is greatly affirmed on the understanding about pathogenesis of AD up to now. Hence, it will play a key role in the treatment of AD if the generation of Aβ could be suppressed. Although many drugs targeting Aβ could not get expected results in the clinical trials, aducanumab, which reduces the number of amyloid plaques present in the brain developed by Biogen and Eisai, is recently submitted to U.S. Food and Drug Administration (FDA) for the approval as the first antibody for mild-middle stage of AD. Therefore, drugs targeting Aβ production worth further investigation and inhibition of BACE1 activity could block one of the earliest pathologic events in AD.

Although we have found the anti-acetylcholinesterase activity of the extract from G. delavayi, there is a large consensus in that the failure to find such a drug for AD is possibly due...
to the multifactorial nature of AD. This is why molecules acting only on one single target is not suitable for AD therapy, and therefore multi-target-directed ligand strategy in treating AD is of potential.

BACE1 works as a rate-limiting enzyme in the production of Aβ. We investigated whether G. delavayi had anti-BACE1 action in APP/PS1 CHO cells. The results show that the chloroform extract of G. delavayi treatment of APP/PS1 CHO cells did not change APP protein levels but decreased the generation of Aβ42 and Aβ40 and the chloroform extract of G. delavayi inhibited the activity of BACE1, which is involved in APP processing. When compared with positive control AZD3293, the chloroform extract of G. delavayi suppress the activity of BACE1 significantly. AZD3293, has shown satisfactory pharmacokinetics and encouraging clinical data in ongoing studies.

Most BACE1 inhibitors not only block BACE1 activity but also are potent inhibitors of related members of the pepsin family of aspartyl proteases, particularly β-site APP cleaving enzymes 2 (BACE2) and cathepsin D. BACE2 is the closest homologue of β-Secretase and is present in the human brain and other tissues and its active-site binding pattern is very similar to that of β-Secretase. On the other hand, cathepsin D is an aspartic protease ubiquitously present in the lysosomes and endosomes of nearly all human cells. It plays a critical role in cellular protein catabolism and the high abundance of this enzyme in the human body makes it a potential trap for β-Secretase inhibitors that lack good cathepsin D selectivity. Therefore a good β-Secretase inhibitor drug candidate needs to be large enough to bind enzymes related to Aβ production with high affinity, while the small differences in the structures between the binding sites of these aspartic proteases and molecules can be explored as the selectivity of the candidates. Luckily, the result shows that the extract does not significantly inhibit BACE2 or cathepsin D either, when compared with the positive control -AZD3293 and HY-P0018. These agents are reported to have a good selectivity on BACE2 and inhibit cathepsin D, respectively.

In addition to inhibiting the production of Aβ peptide, it is also very important to promote the degradation of Aβ oligomers for AD treatment. NEP is a zinc-dependent axonal and synaptic membrane metallopeptidase, which is widely expressed in cortical neurons. NEP overexpression can both prevent and clear Aβ deposits in mouse models of AD, and is considered to be a rate limiting step in the Aβ degradation processing and is a potential target for the treatment of AD. This enzyme degrades misfolded and entangled Aβ peptides in the brain. Its deficiency is associated with increased Aβ accumulation in the brain and leads to deposition of amyloid-like structures in vivo as well as with signs of AD-like pathology and with behavioral deficits when under physiological APP expression. Our study has also obtained the result that the extract increased NEP expression, which might indicate that the component(s) in the extract have multi-target action property except inhibiting BACE1. Thus, it is expected that the current study may also provide chemical direction from G. delavayi for the discovery of compounds with multi-target activities in the repression of Aβ pathology.

CONCLUSION

In summary, our results clearly demonstrate that the chloroform extract of G. delavayi significantly attenuates Aβ generation in APP/PS1 CHO cells. The effect is mediated by inhibiting the BACE1 activity and increasing the NEP protein expression. Our study suggests that the chloroform extract of G. delavayi may be of potential for further development for AD.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant nos. 31360082, 31860098).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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