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Benzoate fraction from *Gentiana rigescens* Franch alleviates scopolamine-induced impaired memory in mice model *in vivo*

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5-bromo-2-deoxyuridine (PubChem CID: 6035)
Dimethyl sulfoxide (PubChem CID: 679)
Donepezil (PubChem CID: 3152)
Ethyl acetate (PubChem CID: 8857)
Formamide (PubChem CID: 713)
Hexane (PubChem CID: 8058)
Malondialdehyde (PubChem CID: 10964)
Methanol (PubChem CID: 887)
Scopolamine (PubChem CID: 5184)

ABSTRACT

Ethnopharmacological relevance: *G. rigescens* Franch (Long Dan Cao in Chinese) is a well-known TCM herb. It is clinically used with other drugs for the treatment of brain diseases such as epilepsy, post-herpetic neuralgia in China.
Aim of study: In our previous study, 11 dihydroxybenzoate compounds with NGF mimicking activity from *G. rigescens* Franch were found. In the present study, the neurogenesis and neuroprotection of a mixture of benzoates (n-GS) were investigated in animal level.
Materials and methods: The NGF mimicking activity of n-GS from *G. rigescens* Franch was examined in PC12 cells. The neurogenesis effects of n-GS were investigated in ICR mice with 5-bromo-2-deoxyuridine (BrdU) and neuronal neclei (NeuN) double immunostaining. Furthermore, the neuroprotection effects of n-GS on the memory in a scopolamine (SCO)-induced mouse model were evaluated with animal behavior tests.
Results: The NGF-mimicking function and neurogenesis of n-GS were observed in PC12 cells and in normal mice. Subsequently, we investigated the effects of n-GS on the memory in a SCO-induced mouse model. In Y-maze test, SCO significantly lowered the alternation. This finding was reversed by n-GS and donepezil (DONE). SCO significantly impaired the mice's performance in novel object recognition (NOR) and Morris water maze (MWM) tests. The time spent to explore the novel object was longer in the n-GS- and DONE-treated groups than in the SCO control group. In the MWM test, the escape latency of n-GS- and DONE-treated groups was shorter than that of the SCO control group. Mechanism study showed that SCO significantly reduced superoxide dismutase (SOD) but increased the activities of acetylcholinesterase (AChE) and the levels of malondialdehyde (MDA) in the hippocampus and cerebral cortex, which all can be improved by n-GS and DONE. Additionally, the phosphorylation of type 1 insulin-like growth factor (IGF-1) receptor, extracellular signal-regulated kinase (ERK), and cAMP responsive element-binding (CREB) protein in the hippocampus was significantly up-regulated in the treatment group compared with that in the SCO group.
Conclusions: n-GS could alleviate impaired memory of the SCO-induced mouse model by inhibiting AChE activity and oxidative stress, and regulating the IGF-1R/ERK signaling pathway.

1. Introduction

Alzheimer's disease (AD), a progressive and fatal neurodegenerative disorder, is the most common form of dementia in the elderly (Cummings, 2004). AD patients fail to initially encode new trivial memories and eventually the details of life (Selkoe, 2002). This devastating disease is also accompanied by the loss of cholinergic markers in vulnerable neurons and the degeneration of basal forebrain cortical cholinergic neurons in end-stage AD patients (Mufson et al., 2008). Changes in AChE activities are most strongly correlated with memory loss and cognitive impairments (Araujo et al., 2005). Furthermore, AChE can increase the rate of fibrillation by binding amyloid-β-associated proteins as potent amyloid-promoting factors (Inestrosa et al., 1996). Thus, the cholinergic hypothesis led to the development of clinically effective therapeutics for AD, such as DONE, rivastigmine, and galantamine. However, these drugs can still cause severe side effects, resulting from the peripheral cholinergic system activation or

Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase; BrdU, 5-bromo-2-deoxyuridine; CREB, cAMP-response element binding protein; DG, dentate gyrus; DMSO, dimethyl sulfoxide; DONE, donepezil; ERK, extracellular signal-regulated kinase; ICR, Institute of Cancer Research; IGF-1, type-1 insulin like growth factor; MDA, malondialdehyde; MWM, Morris water maze; NeuN, Neuronal Nuclei; NGF, nerve growth factor; NOR, novel object recognition; SCO, scopolamine; SOD, superoxide dismutase; TCM, traditional Chinese medicine; T-SOD, Total superoxide dismutase

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hepatotoxicity of these drugs (Lahiri et al., 2003). Thus, alternative anti-dementia therapies should be developed.

Gentiana, containing about 400 species, is the largest genus in Gentianaceae family. Extracts from *Gentiana* species have been used to treat poor appetite and digestive problems (Suyama et al., 2013; Wang et al., 2013). *Gentiana rigescens* (Long Dan Cao) grows in Southwest China and is a well-known herb in traditional Chinese medicine (TCM). Meanwhile, *G. rigescens* Franch is clinically used with other drugs for the treatment of brain diseases such as epilepsy (Hu et al., 2012), postherpetic neuralgia (Shi et al., 2007) in China. In Chinese herbal prescription, it was also used for the treatment of phlegm and heat blockage type acute cerebral hemorrhage (Wu, 2014). By PC12 cells bioassay system, our lab reported 11 new alkyl 2,3-dihydroxybenzoates from *G. rigescens*. These compounds all belong to a novel class of neuritogenic substances *in vitro* (Gao et al., 2010a, 2010b). We used a mixture of benzoates (n-GS) to test their abilities to regulate learning and memory.

Neurogenesis is important for cognitive function involving the hippocampus. Cognitive function may be improved through enhanced proliferation and differentiation of neuronal progenitors (Zonis et al., 2015) and hippocampus is the important region for learning and memory. Meanwhile, AD is characteristics for the loss of learning and memory. Emerging evidence indicated that loss of neurons is related to dementia. Thus, researchers attempted to increase the neural cells in hippocampus as a novel therapeutic strategy for prevention or treatment of AD (Miller and Kaplan, 2012). Furthermore, many herbal medicines, such as *Rosa damascena* extract showed the neurogenesis and synaptogenesis effect in mice also had ability to improve memory in AD (Esfandiary et al., 2014). Hence, the area of herbal medicines, which had neurogenesis function, showed increase promising for new therapies of AD.

SCO, a muscarinic ACh receptor (MAChR) antagonist, can block the cholinergic function of the central nervous system by targeting M1AChR and M2AChR. SCO can induce anterograde memory impairment, particularly short-term memory and learning acquisition (Lee et al., 2014). Furthermore, SCO can significantly increase the levels of AChE, MDA in the cortex and hippocampus, and oxidative stress in the brain (Tao et al., 2014; Jeong et al., 2008). Oxidative damage triggers the pathogenesis and cognitive disturbances in AD (Ding et al., 2007). AD is highly related to cholinergic deficits and intracellular oxidative stress; thus, SCO-induced AD model is a valuable animal model for screening anti-AD drugs.

In the present study, we conducted animal behavior tests to examine whether n-GS can improve cognitive impairment and memory deficits. To elucidate the mechanism of action of n-GS, we investigated the AChE inhibitory property and antioxidant activities of n-GS. The activation of extracellular signal-regulated kinase (ERK) signaling pathway plays a critical role in memory reconsolidation. Additionally, ERK can couple of CREB phosphorylation at the Ser133 site. CREB is widely implicated in many forms of neuronal plasticity and learning memory (Lv et al., 2015; Hall et al., 2001). Thus, we investigated the changes in the IGF-1R/ERK/CREB signaling pathway in the hippocampus and cerebral cortex. We reported herein that n-GS can alleviate the impaired memory in SCO-induced mice by inhibiting AChE, preventing oxidative stress and increasing the phosphorylation of IGF-1R/ERK/CREB signaling.

2. Material and methods

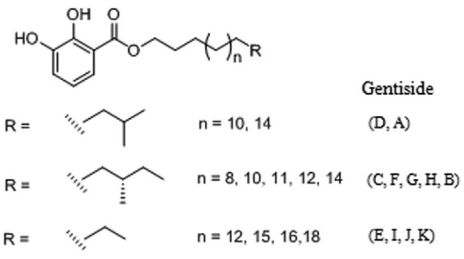
2.1. Preparation of n-GS

Dried roots of *G. rigescens* which were produced in Guizhou Province, China, were collected in October 2013, and they were purchased from HuQingYuTang Pharmacy in Hangzhou, Zhejiang Province, China. Samples were first powdered, and the extracts were obtained within 2 days at room temperature by using 95% methyl alcohol (3 L) with stirring. The extracts were then partitioned between *n*-hexane and 80% aqueous MeOH. The active *n*-hexane layer was concentrated to obtain 675 mg of the dried samples. This layer was chromatographed on silica gel (200–300 mesh, Yantai Chemical Industry Research Institute) and eluted with *n*-hexane/EtOAc (90:10, 85:15, 80:20, 70:30, and 50:50) to yield 32 fractions. According to the TLC results, the gentiside-containing fractions (eluted with *n*-hexane/MeOH 80:20) were combined to yield n-GS (Fig. 1A). The reversed-phase HPLC was used to confirm the percentage of each compound in n-GS as shown in Fig. 1B. This result was consistent with those reported in literature (Gao et al., 2010a, 2010b). We prepared an adequate amount of n-GS prior to the subsequent experiments.

2.2. Nerve growth factor (NGF) mimicking activity bioassay in PC12 cells

NGF mimicking activity was tested on PC12 cells by using the methods described in our previous research (Yang et al., 2014). PC12 cells were seeded in a 24-well microplate (20,000 cells per well) and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, and premixed antibiotics at 37 °C in 5% CO_2. For the bioassay test, the medium was replaced after 24 h with 1 mL of serum-free DMEM containing 0.5% dimethyl sulfoxide (DMSO) and a test sample. Morphological changes in the cells were observed under a microscope after 24 and 48 h. A positive cell was characterized by a longer neurite outgrowth than the diameter of the cell body. A total of 100 cells were randomly counted from an

(A)



(B)

Gentiside	Percentage in n-GS (%)	w/w (%)	Gentiside	Percentage in n-GS (%)	w/w (%)
A	22.27	0.000750	G	4.93	0.000166
B	32.40	0.001091	H	4.34	0.000146
C	6.03	0.000203	I	0.50	0.000017
D	1.11	0.000037	J	21.17	0.000713
E	1.06	0.000036	K	4.96	0.000167
F	1.23	0.000041	Total	100	0.003368

Fig. 1. Chemical structures and the main components of n-GS in crud drug were shown in (A) and (B). The relative ratio in the active fraction and w/w (%) content of the single benzoate in the crude drug were used to represent ingredient.