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Improving the acetylcholinesterase inhibitory effect of *Illigera henryi* by solid-state fermentation with *Clonostachys rogersoniana*

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***Illigera henryi*, an endemic traditional Chinese medicine, contains abundant aporphine alkaloids that possess various bioactivities. In the present study, tubers of *I. henryi* were fermented by several fungi, and the acetylcholinesterase (AChE) inhibitory activities of non-fermented and fermented *I. henryi* were measured. The results showed that the fermentation of *I. henryi* with *Clonostachys rogersoniana* 828H2 is effective for improving the AChE inhibitory activity. A key biotransformation was found during the *C. rogersoniana* fermentation for clarifying the improvement of the AChE inhibitory activity of *I. henryi*: (*S*)-actinodaphnine (1) was converted to a new 4-hydroxyaporphine alkaloid (4*R*,6a*S*)-4-hydroxyactinodaphnine (2) that possessed a stronger AChE inhibitory activity, with an IC_{50} value of 17.66 ± 0.06 μM. This paper is the first to report that the pure strain fermentation processing of *I. henryi* and indicated *C. rogersoniana* fermentation might be a potential processing method for *I. henryi*.**

[**Key words:** *Illigera henryi*; *Clonostachys rogersoniana*; Acetylcholinesterase inhibitory activity; Fermentation; (4*R*,6a*S*)-4-Hydroxyactinodaphnine]

Illigera henryi W. W. Sm. is a liana belonging to genus *Illigera* (Hernandiaceae) that mainly grows in Guangxi and Yunnan, China (1). Its tubers are frequently used as a traditional Chinese medicine (TCM) for treating cough and tuberculosis and providing sedation. The chemical constituents of its congener plants such as *Illigera luzonensis* and *Illigera aromatica* are aporphine alkaloids (2–5), phenolic acids, and steroids (6), which are reported to possess various bioactivities such as cholinesterase inhibitory activity, vasorelaxation activity (2), cytotoxic activity (4,5), and anti-platelet aggregation activity (6). Our previous research revealed that aporphine alkaloids are significant chemical constituents of *I. henryi* (7).

Solid-state fermentation has been used to produce secondary metabolites from folk medicinal plants since approximately 4000 years ago in China. In ancient China, natural fermented herbal medicines (HMs) such as *Rhizoma Pinelliae Fermentata*, *Semen Sojae Praeparatum*, and *Massa Medicata Fermentata* were very popular. In modern times, the fermentation of HMs using pure strains has become increasingly reliable and accepted. In recent years, fermented HMs (FHMs) have garnered increasing attention in East Asia, especially in Korea and Taiwan. Some FHMs exhibit stronger biological activities or higher bioavailability than the original medicines. For example, Palaniyandi et al. (8) reported a transformation of ginsenoside Rb1 to Rg3 and Rh2 by the fermentation of *Panax ginseng* C. A. Meyer with *Lactobacillus paracasei* subsp. tolerans MJM60396. Fermenting *Radix astragali* with *Bacillus subtilis* could similarly significantly stimulate the biosynthesis of type I procollagen in a dose-dependent manner (9). The fermentation of *Curcuma longa* L. with *Aspergillus oryzaeis* could effectively prevent CCl_4-induced hepatic damage in rats (10). Wang et al. (11) reported that the roots of *Angelica dahurica* fermented with *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, and *Lactobacillus brevis* showed stronger tyrosinase inhibitory and antioxidant activities than the non-fermented material.

In the present study, tubers of *I. henryi* were fermented with several fungi. The acetylcholinesterase (AChE) inhibitory activities of the non-fermented and fermented *I. henryi* were measured to screen the processing method for improving the AChE activity, and the fermentation time and temperature were also discussed. This paper presents a novel approach to improve the AChE inhibitory activity of *I. henryi*.

MATERIALS AND METHODS

General The optical rotation was tested using a Jasco P-1020 digital polarimeter (Jasco, Tokyo, Japan). Electronic circular dichroism (ECD) spectra were measured using a Chirascan circular dichroism spectrometer (Applied Photophysics, Ltd., Leatherhead, UK). Melting points (m.p.) were determined by an XRC-1 Melting Pointing Apparatus (uncorrected, Optical Instrument Factory of Sichuan University, Chengdu, China). Nuclear magnetic resonance (NMR) spectra including 1D/2D NMR were recorded using a Bruker Avance 400 MHz spectrometer (Bruker, Karlsruhe, Germany) with a 5-mm NMR tube, using TMS as the internal reference. High-resolution electrospray ionization mass spectrometry (HRESIMS) was conducted using an Agilent G3250AA spectrometers (Agilent, Santa Clara, CA, USA). Thin-layer chromatography (TLC) was visualized by spraying with a modified Dragendorff's reagent. The acetonitrile used for the HPLC analysis was purchased from Fisher Scientific Co., Ltd. (Fair Lawn, NJ, USA).

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Plant material The tubers of *I. henryi* were collected in Wenshan, Yunnan, China, in December 2015 and identified by Assistant Professor Shuda Yang at the College of Pharmacy, Kunming Medical University, Kunming, China. A voucher specimen (2015-IH-01) has been deposited in the School of Chemical Science and Technology, Yunnan University, Kunming, China.

Microorganisms All strains including *Penicillium vancouverense* C2-1, *Penicillium chrysogenum* WT-12, *Arthrinium phaeospermum* M18-7, *Alternaria alternate* YJ-19, *Geomyces luteus* P18-5, *Penicillium cordubense* WT-7, *Aspergillus tubingensis* WT-5, *Clonostachys rogersoniana* 828H2, *Colletotrichum* sp. YJ-20, *P. chrysogenum* YJ-1, *Trichophyton verrucosum* YM3096, *Penicillium virgatum* WT-45, *Phyllosticta capitalensis* YJ-J4, *Mucor racemosus* f. racemosus WT-1, *Geomyces pannorum* WT-27, *Verticillium zaregamsianum* WT-28, *Acremonium furcatum* WT-39, *Alternaria* sp. YJ-18, *Aspergillus versicolor* WT-49, *Scytalidium lignicola* M1-11 were obtained from the Yunnan Institute of Microbiology, Yunnan Province, China.

Herbal fermentation procedure *C. rogersoniana* 828H2 was activated in a PDA (1 L water, 200 g potato, 20 g dextrose, and 15 g agar) slant culture medium and stored in a constant-temperature incubator at 28°C for 6 days. The fermentation culture medium consisted of 10 g powder of *I. henryi* was infiltrated with 40 mL water and sterilized at 121°C for 30 min. After cool down to room temperature, the fermentation medium was inoculated with the mature fungi and cultivated in a constant-temperature incubator at 28°C for 30 days. The blank control was treated the same as the fermented *I. henryi* in the absence of the fungus.

The experiments of the effects of the fermentation time and temperature were carried out at the same conditions as above. Briefly, the fermentation culture medium (*I. henryi*) was infiltrated with 40 mL water and sterilized at 121°C for 30 min. Then the fermentation medium was inoculated with the mature fungi. The experiments of cultivations at different temperatures for 30 days and cultivations at 28°C for different time were used to investigate the effects of temperature and fermentation time, respectively.

Extraction and isolation The non-fermented *I. henryi* (20 g), *C. rogersoniana*-fermented *I. henryi* (20 g), and blank control (20 g) were each immersed in 200 mL methanol and thoroughly extracted 3 times for 30 min at room temperature by ultrasonic extraction. The extracts were decanted and filtered at room temperature and then concentrated in a rotary evaporator to obtain two extracts: NFIH (1.62 ± 0.11 g, non-fermented *I. henryi*) and CFIH (1.67 ± 0.05 g, *C. rogersoniana*-fermented *I. henryi*).

NFIH was subjected to silica gel CC [chloroform-methanol (10:1-8:1)] and further separated by Sephadex LH-20 (methanol) to yield **1** (68.0 mg). CFIH was chromatographed over a silica gel column [chloroform-methanol (30:1-10:1)] to give fractions A-C. Fraction A (95.0 mg) was purified by a Sephadex LH-20 (methanol) to afford **2** (55.0 mg).

Fermentation using 1 as substrate *C. rogersoniana* 828H2 was activated by the same activation process as mentioned above. Potato dextrose broth (PDB) medium (1 L water, 200 g potato and 20 g dextrose) and potato cubes (50 g) were each sterilized at 121°C for 30 min and inoculated with the mature fungi and compound **1** (10 mg) in a constant-temperature incubator at 28°C for 7 days (PDB medium) and 30 days (potato medium). The blank controls of the PDB medium and potato medium were treated the same but in the absence of compound **1**.

AChE inhibitory activity The AChE inhibitory activity was determined using the Ellman method described in the literature (12). Tacrine was used as the positive control.

HPLC analysis All samples (non-fermented and fermented *I. henryi*) and standards were filtered through a 0.45-μm filter before injection into an Agilent 1200 series HPLC system equipped with two Agilent G1310A unit pumps, an Agilent G1315D diode array detector, an Agilent G1322 degasser, an Agilent G1329A autosampler, an Agilent Zorbax Eclipse XDB-C18 (250 × 4.6 mm i.d., 5 μm) column (SN: USNH047155), and an Agilent Chemstation. A gradient elution system consisting of solvents A (water containing 0.1% phosphoric acid and 0.4% triethylamine) and B (acetonitrile) was used for the analysis, and the gradient program was as follows: 0–5 min, 20%–40% solvent B; 5–15 min, 40–50% solvent B; 15–20 min, 50% solvent B. The peaks were confirmed by the retention time at 280 nm. The flow rate was 1.0 mL/min, the column temperature was set to 25°C, and the injection volume was 10 μL. Each sample was analyzed in triplicate, and the contents of products were determined from the corresponding calibration curves (Table S1)

Calculation The theoretical ECD spectra of 4-hydroxyactinodaphnine (**2**) were calculated using the Gaussian Program by the Yunnan Electronic Computing Center. Four possible geometries, (4*R*,6a*R*), (4*R*,6a*S*), (4*S*,6a*R*), and (4*S*,6a*S*), were previously optimized by the DFT method at the B3LYP/6-31G(d,p) level, and the excitation energies and rotational strengths were calculated using TDDFT at the B3LYP/6-31G(d,p) level in methanol with the PCM model (13,14). Afterwards, the ECD spectra were generated using SpecDis (15–17).

Spectroscopic data (*S*)-Actinodaphnine (**1**): Pale yellow amorphous powder; $[\alpha]^{25}_{D}$ + 44.5 (*c* 0.23, MeOH); HRESIMS *m/z* 312.1229 (calculated for $C_{18}H_{18}NO_4$ 312.1230); ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

(4*R*,6a*S*)-4-Hydroxyactinodaphnine (**2**): Pale yellow amorphous powder; $[\alpha]^{25}_{D}$ + 11.7 (*c* 0.17, MeOH); HRESIMS *m/z* 328.1177 (calculated for $C_{18}H_{18}NO_5$ 328.1179); ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

TABLE 1. ^{1}H and ^{13}C NMR spectroscopic data for compound **1** and **2**.

Position	(*S*)-Actinodaphine (**1**)		(4*R*,6a*S*)-4-Hydroxyactinodaphine (**2**)	
	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)
1	149.7 s		149.9 s	
1a	117.7 s		117.5 s	
2	143.7 s		145.1 s	
3	107.6 d	6.56 s	108.6 d	6.82 s
3a	121.4 s		121.8 s	
4	26.4 t	3.27 dd (1.6, 4.0) 2.75 d (13.2)	63.9 d	4.81 t (2.0)
5	42.6 t	3.6 dd (4.0, 13.2) 3.49 dd (4.0, 13.2)	49.9 t	3.44 dd (12.8, 2.0) 3.59 dd (12.8, 2.0)
6a	54.3 d	4.17 dd (4.0, 13.6)	54.5 d	4.25 dd (14.0, 5.2)
7	33.7 t	3.14 dd (4.0, 13.6) 2.98 dd (4.0, 13.6)	33.4 t	2.94 t (14.0) 3.02 dd (14.0, 5.2)
7a	122.9 s		122.8 s	
8	116.1 d	6.68 s	116.2 d	6.75 s
9	147.7 s		148.1 s	
10	148.3 s		148.3 s	
11	112.1 d	7.54 s	112.1 d	7.62 s
11a	126.6 s		126.7 s	
OCH_2O	102.6 t	5.92 s 6.08 s	102.9 t	6.02 s 6.17 s
10-OCH_3	56.5 q	3.79 s	56.5 q	3.84 s

The nucleotide sequence data of *C. rogersoniana* 828H2 has been deposited in GenBank (accession number KT625993).

RESULTS AND DISCUSSION

AChE inhibitory effects of non-fermented and fermented *I. henryi* Twenty fungi were initially screened for their ability to improve the biological activity of *I. henryi*. After 30 days of fermentation, the AChE inhibitory activities of non-fermented and fermented *I. henryi* (NFIH and FIH) were determined. The results showed *C. rogersoniana* 828H2 fermented *I. henryi* (CFIH) possessed an AChE inhibition ratio of 42.20 ± 1.20 at a concentration of 200 μg/mL (Fig. 1), while the NFIH and the other nineteen strains FIH did not exhibit obvious AChE inhibitory effects, suggesting that fermenting *I. henryi* using the fungus *C. rogersoniana* might be feasible for improving its AChE activity.

TLC and HPLC analyses The changes of the bioactivities of TCMs during microbial fermentation are frequently ascribed to

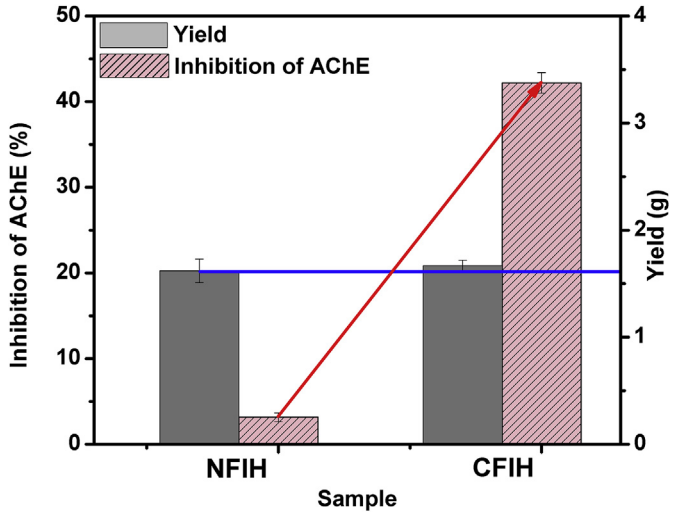

FIG. 1. The yields and inhibitions (%) of AChE of non-fermented and *C. rogersoniana* fermented *I. henryi* (NFIH and CFIH).