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Original article

Antibacterial active compounds from *Hypericum ascyron* L. induce bacterial cell death through apoptosis pathway

Xiu-Mei Li [a, 1], Xue-Gang Luo [a, *, 1], Chuan-Ling Si [c, d], Nan Wang [a], Hao Zhou [a], Jun-Fang He [a], Tong-Cun Zhang [a, b, *]

[a] Key Lab of Industrial Fermentation Microbiology of the Ministry of Education, Tianjin Key Lab of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin, 300457, PR China
[b] Institute of Biology and Medicine, Wuhan University of Science and Technology, Wuhan, 430000, PR China
[c] Tianjin Key Laboratory of Pulp & Paper, Tianjin University of Science & Technology, Tianjin, 300457, PR China
[d] State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin, 150040, PR China

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ABSTRACT

Hypericum ascyron L. has been used as a traditional medicine for the treatment of wounds, swelling, headache, nausea and abscesses in China for thousands of years. However, modern pharmacological studies are still necessary to provide a scientific basis to substantiate their traditional use. In this study, the mechanism underlying the antimicrobial effect of the antibacterial activity compounds from *H. ascyron* L. was investigated. Bioguided fractionation of the extract from *H. ascyron* L. afforded antibacterial activity fraction 8. The results of cup plate analysis and MTT assay showed that the MIC and MBC of fraction 8 is 5 mg/mL. Furthermore, using Annexin V-FITC/PI, TUNEL labeling and DNA gel electrophoresis, we found that cell death with apoptosis features similar to those in eucaryon could be induced in bacteria strains after exposure to the antibacterial activity compounds from *H. ascyron* L. at moderate concentration. In addition, we further found fraction 8 could disrupt the cell membrane potential indicate that fraction 8 exerts pro-apoptotic effects through a membrane-mediated apoptosis pathway. Finally, quercetin and kaempferol 3-*O*-*β*-(2″-acetyl)-galactopyranoside, were identified from fraction 8 by means of Mass spectrometry and Nuclear magnetic resonance. To our best knowledge, this study is the first to show that Kaempferol 3-*O*-*β*-(2″-acetyl)-galactopyranoside coupled with quercetin had significant antibacterial activity via apoptosis pathway, and it is also the first report that Kaempferol 3-*O*-*β*-(2″-acetyl)-galactopyranoside was found in clusiacea. Our data might provide a rational base for the use of *H. ascyron* L. in clinical, and throw light on the development of novel antibacterial drugs.

1. Introduction

Antimicrobial resistances against human pathogenic microorganisms have developed to be more and more serious due to the indiscriminate use of antimicrobial drugs in the treatment of infectious diseases. This situation, the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections [1–3] forced scientists into looking for new antimicrobial substances from various sources. Lots of evidence showed that plants, especially traditional herbs, represent potential source of new anti-infective agents [1]. They could markedly mitigate infectious diseases, but lack adverse side effects which are often associated with traditional antimicrobial agents, including hypersensitivity, allergic reaction, and immunosuppression [4–6]. In particular, Hypericum is one important genus of traditional Chinese herb medicine, which could produce secondary metabolites with antimicrobial properties [7].

Among 484 *Hypericum* L. (Guttiferae/Hypericaceae) species which are widespread in warm temperate areas throughout the world, only *Hypericum perforatum* is widely used in official medicine [8]. *Hypericum ascyron* L. is a perennial herbaceous plant genus of the Guttiferae family. It is commonly found in Japan and the

* Corresponding authors. Key Lab of Industrial Fermentation Microbiology of the Ministry of Education, Tianjin Key Lab of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin, 300457, PR China.
E-mail addresses: luoxuegang@hotmail.com (X.-G. Luo), tony@tust.edu.cn (T.-C. Zhang).
[1] The first two authors contributed equally to the project.

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northeast, the basin of Yellow River and Yangtze River of China. It common names include Hong-Han-Lian, Huang-Hai-Tang, and Hunan-Lian-Qiao. The aerial parts of the plant have been used as a traditional Chinese medicine for the treatment of wounds, swelling, headache, nausea, abscesses, abnormal menstruation and promoting lactation [9]. Recent studies have revealed many mechanisms underlying the pharmacological activities of *H. ascyron* L., including of histamine-release inhibitory [10], anti-inflammatory and analgesic effects [11], anti-oxidant activity [12] glucosidase inhibitory, anti-diabetic activity [13], and anticancer [14]. However, the antibacterial activity of *H. ascyron* L. was not reported.

Plants are widely accepted as good sources of novel antimicrobial agents. Screening of antimicrobial activities to find which types of bacteria are susceptible to plant extracts is useful, however the investigation of underlying mechanism is also crucial for drug development. To explore the possible antibacterial activity compounds and antibacterial mechanism of *H. ascyron* L.. Firstly, the crude extract of *H. ascyron* L. was prepared and its function on the proliferation of bacterial cells was analyzed. Secondly, to identify the compounds which are responsible for the antibacterial activity, a bioassay-guided fractionation was employed using preparative HPLC, and the structural characterization of the antibacterial compounds was analyzed using NMR and electrospray ionization tandem mass spectrometry (ESI/MS). Thirdly, biochemical methods were utilized to explain the bacterial cell death mechanism.

2. Materials and methods

2.1. General experimental procedures

Agela Akasil C18 column (250 mm × 30 mm i.d., 5 μm; Agela Technologies, Inc; China) and Kromasil C18 column (250 × 4.6 mm i.d.; 5 μm; AkzoNoble, Sweden) were used for column chromatography. For preparative HPLC, a preparative-HPLC system (Agilent Technologies, Inc; USA) consisting of an Agilent 1200 series pump, manual injector, binary pump, and a 1200 series variable-wavelength UV-VIS detector was used for all analyses. For a analytical HPLC system (Agilent Technologies, Inc; USA) consisting of an Agilent 1100 series pump, manual injector, a binary pump and a 1100 series variable-wavelength UV-VIS detector was used for all analyses. NMR spectra (^{1}H, ^{13}C, COSY, HMBC, HSQC) were recorded in DMSO-d_6 spectrometer with a Bruker Avance III 400 MHz equipped with a cryoprobe and using TMS as the internal reference. Mass spectrometry (MS) was carried out using a micromass ESI instrument (Agilent Technologies, Inc; USA) using ESI-ionization in positive mode.

2.2. Plant material

All the samples of *H. ascyron* L. were collected from Hailaer (China). All specimens, which were authenticated by Ya-hong Sun (Genhe city of Inner Mongolia agriculture and animal husbandry bureau, Genhe, PR China), were dried in the shade until the weight remained constant.

2.3. Microbial strains

The antimicrobial activity of *H. ascyron* L. extract and its fractions were tested against the following microorganisms: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus aureus* (ATCC 25923) and *Micrococcus luteus* (ATCC 10240). All strains were provided by China General Microbiological Culture Collection Center (CGMCC) and maintained in Mueller-Hinton agar (Hopebio, China) and stored at −20 °C.

2.4. Antibacterial activity evaluation

The antimicrobial efficacy of all the samples was evaluated using cup plate method. Mueller-Hinton agar plates were prepared by pouring 10–15 mL of the medium into each sterile Petridish and were allowed to set at room temperature. The bacterial cell suspension was standardised to the optical density of 0.1 at 600 nm using a spectrophotometer. Thus final concentration of microorganisms in the inoculum was adjusted to 10^5 CFU/mL and was inoculated over the surface of agar medium using a sterile cotton swab. The Oxford cups were placed in each plate. Each Oxford cup was added 200 μL of the designed concentration of *H. ascyron* L. extract and fractions. The petri plates were then incubated at 37 °C for 18 h.

2.5. Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used for measuring the proliferation of bacterial cells. Briefly, bacterial cells (10^5 CFU/mL) were inoculated into Mueller-Hinton broth at 200 μL/well in 96-well microtiter plates. Two fold serial dilutions of *H. ascyron L.* extract were added to wells containing bacterial cells. After 24 h of incubation at 37 °C, each concentration was assayed in triplicate (n = 3). Twenty-four hours later, 10 μL of the MTT (5 mg/mL) reagent was added to each well and the plates were incubated for 4 h at 37 °C. Then, DMSO (100 μL) was added to terminate the reaction, and the plate was shaken slightly to redissolve the crystals formed. The absorbance of each well was measured using Synergy 4 microplate reader (BioTek Instruments, Winooski, VT, USA). All the results were expressed as the inhibition ratio of cell proliferation calculated as [(A-B)/A] ×100%, where A and B were the average numbers of viable cells of the control and samples, respectively.

MBC, defined as the minimum concentration required to killing 99.9% of a bacteria inoculums, was determined by reinoculating 20 μL of each culture medium from the microtiter plate wells onto Mueller-Hinton agar plates. After 18 h of incubation at 37 °C, MBC value was determined by visually inspecting the agar plates for bacterial growth. MIC and MBC measurements were performed at least in triplicate (n = 3).

2.6. Bioassay-guided isolation

The aerial parts of the dried plants were harvested and then selected through a 60 mesh sieve. Each sample powder (1.00 g) was accurately weighed and extracted using 30 mL (60%, v/v) ethanol-water solution by ultrasonic extraction for 30 min at 30 °C [14]. The extract obtained was centrifuged for 10 min at 5000 rpm with a Hermle Z206A centrifuge (Denville Scientific Inc., USA).The extract was collected and filtered through a membrane filter with a pore size of 0.22 μm (Agela Technologies, Inc; China). The extract was separated on an Agela Akasil C18 column (250 mm × 30 mm i.d., 5 μm). Acetonitrile and water with 0.1% acetic acid were used as mobile phases. The gradient of elution program was as follows: from 0 to 5 min, acetonitrile followed a linear change from 5% to 10%; from 5 to 10 min, acetonitrile linearly changed from 10% to 15%; from 10 to 15 min, solvent A linearly changed from 15% to 17%; from 15 to 30 min, acetonitrile was isocratic at 17%; from 30 to 60 min, acetonitrile linearly changed from 17% to 25%; from 60 to 90 min, acetonitrile linearly changed from 25% to 45%. The flow rate was at 15 mL/min with a 10 mL injection volume, The absorbance was monitored at 275 nm [14] to give 10 fractions (as shown in Fig. 1(A)) based on pre-HPLC detection peaks at different retention time. Each fraction was tested for antibacterial activity and the