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Three new anti-HBV active constituents from the traditional Chinese herb of Yin-Chen (*Artemisia scoparia*)

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ABSTRACT

Ethnopharmacological relevance: Yin-Chen is a famous traditional Chinese medicine (TCM) in China for the treatment of acute and chronic hepatitis. Two species, namely *Artemisia scoparia* and *Artemisia capillaris*, are documented in Chinese Pharmacopoeia as the authentic resources for Yin-Chen. Previous investigation has proved that chlorogenic acid analogs and phenolic acids are two main types of the anti-HBV active constituents of *A. capillaris*. However, there is no investigation concerned with the anti-HBV components of *A. scoparia*.

Aim of the study: The aim of the present study is to recognize the new anti-HBV constituents of *A. scoparia* by detailed LCMS analyses.

Materials and methods: LCMS and bioassay-guided fractionation on the active part of *A. scoparia* led to the isolation of three new compounds. Their structures were determined by detailed spectroscopic analyses. Anti-HBV assay involving inhibition on HBsAg and HBeAg secretions and HBV DNA replication were performed *in virto* on HepG 2.2.15 cell line.

Results: The 90% ethanol extract of *A. scoparia* was revealed with anti-HBV activity for the first time, which was further separated into several fractions by column chromatography. Fr. D-4 was revealed with the highest anti-HBV activity, from which three new compounds including one unusual 4-pyridone glucoside (**1**) and two polyacetylene glucosides (**2**–**3**) were isolated under the guidance of LCMS analyses. Compounds **1**–**3** exhibited activity against the secretions of HBsAg and HBeAg, and HBV DNA replication. In particular, compounds **2** and **3** inhibited HBV DNA replication with IC_{50} values of 0.07 ± 0.04 and 0.012 ± 0.05 mM, with SI values of 23.6 and 17.1, respectively. Based on the MS/MS experiment, the fragmentation pathways of **1** in both positive and negative modes, and **2** and **3** in negative mode were proposed. The ion pairs of 388–208 (positive) and 432–206 (negative) for **1**, 503–341 (negative) for **2**, and 503–203 (negative) for **3**, could be recognized as their respective diagnostic ions.

Conclusions: The first time investigation on the anti-HBV constituents of *A. scoparia* yielded three new active compounds, which will provide valuable information for understanding the ethnopharmacological usage of Yin-Chen, as well as the chemical difference with *A. capillaris*.

1. Introduction

Hepatitis B virus (HBV) infection as a major cause of viral hepatitis is a severe health problem worldwide, especially in China. More than two billion people have been infected with HBV in their lives, 350 million of which are living as long-term HBV carriers. The current treatment strategies involving vaccines, interferons and nucleosides are unsatisfied due to drug-resistance and adverse side effects (Bhattacharya and Thio, 2010; Liaw et al., 2010; Mirandola et al., 2011). Traditional Chinese medicines (TCMs) have been used for medicinal purposes in China for thousands of years, and are fascinating sources for anti-HBV drug discovery. Many anti-HBV candidates with diverse structures and mechanisms have been reported from diverse TCMs, such as wogonin from *Scutellaria baicalensis*, chlorogenic acid analogs from *Artemisia capillaris*, oxysophocarpine from *Sophora flavescens*, and swerilactones from *Swertia mileensis* (Geng et al., 2013; Li et al., 2013; Wang et al., 2011; Zhang and Wang, 2014). Yin-Chen as a famous TCM has been used to treat acute and chronic hepatitis in China for a long time, which could be traced back to the first Chinese dispensatory, "Shen-Nong-Ben-Cao-Jing". Furthermore, many formulae containing Yin-Chen are also widely used for

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hepatoprotective, choleretic, and diuretic purposes in clinic. Two species, namely *Artemisia scoparia* Waldst. et Kit. (Asteraceae) and *Artemisia capillaris* Thunb. (Asteraceae), are documented in Chinese Pharmacopoeia as the authentic resources for Yin-Chen (Chinese Pharmacopoeia Commission, 2010), from which coumarins, polyacetylenes, flavonoids, organic acids and chromones, *etc*. have been revealed as the main constituents (Sharma and Ali, 1996; Wu et al., 2001; Xie et al., 2004). However, the components accounting for their respective anti-HBV property are still disputed. The recent investigation on *A. capillaris* yielded a series of polyacetylenes, coumarins, flavonoids, and chlorogenic acid analogs with anti-HBV activity (Zhao et l., 2014a; Zhao et al., 2014b). In contrast, there is no investigation concerned with the anti-HBV components of *A. scoparia*. Presently, LCMS technique is widely applied in many areas of analytical chemistry due to its high sensitivity and efficiency (Li, et al., 2014; Yang et al., 2014). The Shimadzu UFLCMS-IT-TOF apparatus which is equipped with an electrospray ionization (ESI) source and coupled to ion-trap (IT) and time-of-flight (TOF) mass analyzers is effective for targeted isolation of trace components from natural complex (Geng et al., 2012; Geng et al., 2014). Therefore, the following LCMS guided investigation on the anti-HBV constituents of *A. scoparia* was carried out for better understanding the biological and chemical difference between the two plants of Yin-Chen.

Anti-HBV bioassay on HepG 2.2.15 cell line *in vitro* manifested that 90% ethanol extract of *A. scoparia* showed inhibition on the secretions of HBsAg and HBeAg with inhibition rates of 36.5 ± 8.1 and $25.0 \pm 6.7\%$ (1 mg/mL), and HBV DNA replication with inhibition rate of $49.3 \pm 9.7\%$ (0.25 mg/mL). As a result, the following investigation on the 90% ethanol part yielded three new compounds including one novel 4-pyridone glucoside (**1**) and two polyacetylene glucosides (**2**–**3**) under the guidance of LCMS analyses. The structures of the new compounds **1**–**3** were elucidated by extensive HRESIMS, 1D and 2D NMR, $[\alpha]_D$ and ECD spectroscopic analyses, and quantum calculations by the time-dependent density functional theory (TD-DFT). In this paper, the LCMS guided isolation, structural elucidation and anti-HBV activities of the isolated compounds are discussed.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured through a Jasco model 1020 digital polarimeter (Horiba, Tokyo, Japan). UV spectra were recorded on a Shimadzu UV2401PC spectrophotometer (Shimadzu, Kyoto, Japan). Electronic circular dichroism (ECD) spectra were performed on an Applied Photophysics Chirascan instrument (Agilent, Santa Clara, United States). IR (KBr) spectra were measured on a Bio-Rad FTS-135 spectrometer (Hercules, California, USA). 1D and 2D NMR were recorded on an AVANCE III-600 spectrometer (Bruker, Bremerhaven, Germany). Mass spectra were determined by a LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). Silica gel (200 − 300 mesh) for column chromatography and TLC plates (GF_{254}) were purchased from Qingdao Makall Chemical Company (Makall, Qingdao, China). Sephadex LH-20 for chromatography was obtained from Pharmacia Fine Chemical Co., Ltd. (Pharmacia, Uppsala, Sweden). CHP20P MCI gel (Mitsubishi Chemical Corporation, Tokyo, Japan) was applied for MPLC preparation, which was performed on a Dr-Flash-II MPLC system (Lisui, Suzhou, China). The Chuangxin Tongheng LC3000 apparatus (Beijing Chuangxin Tongheng Science and Technology Co., Ltd., Beijing, China) was used for HPLC preparation on an Agilent Eclipse XDB-C_{18} column (Agilent Technologies, Santa Clara, USA).

2.2. Plant material

The plant material of *A. scoparia* was purchased from Qinghai Fukang Pharmaceutical Group Ltd., Xining, Qinghai Province, China, in August 2013, and authenticated by Dr. Li-Gong Lei (Kunming Institute of Botany, CAS). A voucher specimen (No. 20130801) was deposited in the Laboratory of Anti-virus and Natural Medicinal Chemistry, Kunming Institute of Botany, CAS.

2.3. Acidic hydrolysis

Compounds **1–3** (each 3.0 mg) were individually hydrolyzed by 1 mol/L HCl (2 mL) under reflux for 5 h. After neutralization with $NaHCO_3$ and extraction with $CHCl_3$, the aqueous layer was concentrated and detected by TLC over silica gel ($CHCl_3$–MeOH–H_2O, 60:40:4). The presence of glucose was confirmed by comparison with authentic samples (R_f 0.4). The aqueous part was further purified by Si CC and identified to be D-glucose based on their $[\alpha]_D$ values in MeOH [**1**: +60.9 (*c* 0.05), **2**: +46.0 (*c* 0.06), **3**: +73.1 (*c* 0.06)].

2.4. Anti-HBV assay on HepG 2.2.15 cell line in vitro

The anti-HBV assay was performed according to the previous report (Geng et al., 2011), with tenofovir (Jiangxi Chenyang Pharmaceutial Co., Ltd, China, purity > 97.6%) as the positive control. The inhibition of the secretions of HBsAg and HBeAg was assayed by ELISA method; the inhibition of HBV DNA replication was monitored by the real-time quantitative PCR method; and the cytotoxicity was assessed by the modified MTT method. All the samples were dissolved in DMSO with the final concentration of DMSO in the culture not higher than 2.5‰ (v/v).

2.4.1. Inhibition assay of HBsAg and HBeAg secretions

HepG 2.2.15 cells were seeded in a 48-well microplate at a density of 1×10^4 cells/well and cultured for 72 h at 37 °C with 5% CO_2. The culture was refreshed by fresh medium with/without the tested samples and cultured for additional 72 h. The supernatant was collected and tested for HBsAg and HBeAg levels using the ELISA method. The absorbance (*A*) of each well was measured at 490 nm with a microplate reader (Model 680, Bio-Rad, Inc., USA).

2.4.2. Inhibition assay of HBV DNA replication

HepG 2.2.15 cells were seeded in 24-well plates at a density of 5×10^5 cells/mL and cultured for 2 days. Then the culture medium was replaced by fresh medium with/without the tested samples every other day and cultured for additional 5 days. After removing the supernatant, cells were collected and total DNA was isolated by using the TIANamp Gemomic DNA Kit (TIANGEN, Biotech Co., Ltd., Beijing) following the manufacturer's protocol. The real-time PCR assay was used to detect the HBV DNA.

2.4.3. MTT-based cytotoxicity assay

After removing the supernatant used for HBV antigen secretion assay, the plates were air dried and added with MTT solution (800 mg/mL in phosphates buffer solution, 200 mL/well). After staying at 37 °C for 4 h, the MTT solution was replaced with DMSO (200 mL/well) and incubated at 37 °C for additional 10 min. Then, the solution was mixed by continuous pipetting and transferred to 96-well plates (100 μL per well). The absorbance (*A*) was measured at 490 nm by the automatic plate reader Model 680 (Bio-Rad, Inc., USA).