European Journal of Integrative Medicine 20 (2018) 165–172

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/locate/eujim

Research paper

Sanguisorba officinalis extract, ziyuglycoside I, and II exhibit antiviral effects against hepatitis B virus

Eungyeong Jang[a,b,1], Soyoung Kim[c,1], Na-Rae Lee[c], Hayeon Kim[d], Sooin Chae[e,f], Chang-Woo Han[g], Youngchul Kim[a], Kyung-Tae Lee[e,f], Bum-Joon Kim[h], Kyung-Soo Inn[c,*], Jang-Hoon Lee[a,**]

[a] Department of Internal Medicine, College of Korean Medicine, Kyung Hee University, Seoul 02447, Republic of Korea
[b] Department of Internal Medicine, Kyung Hee University Korean Medicine Hospital, Seoul 02447, Republic of Korea
[c] Department of Fundamental Pharmaceutical Science, Graduate School, Kyung Hee University, Seoul 02447, Republic of Korea
[d] Department of Clinical Korean Medicine, Graduate School, Kyung Hee University, Seoul 02447, Republic of Korea
[e] Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea
[f] Department of Life and Nanopharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea
[g] Department of Internal Medicine, Korean Medicine Hospital, Pusan National University, Yangsan 50612, Republic of Korea
[h] Department of Microbiology and Immunology, Liver Research Institute, Cancer Research Institute and SNUMRC, School of Medicine, Seoul National University, Seoul 03080, Republic of Korea

ARTICLE INFO

Keywords:
Sanguisorba officinalis
Ziyuglycoside I
Ziyuglycoside II
Hepatitis B virus
Genotype C
HepG2.2.15

ABSTRACT

Introduction: *Sanguisorba officinalis* (SO) has been commonly used to relieve dermatitis, burns, intestinal infections, hematemesis, and melena for a long time in China, Korea, and Japan. The aim of this study was to investigate the antiviral effects of 30% ethanol extract of SO (SOE) and its major compounds (ziyuglycoside I and II) against HBV in HepG2.2.15 cells and HepG2 cells transfected with HBV genotype C.

Methods: The antiviral activities of SOE were evaluated by ELISA and quantitative RT-PCR assay in *in vitro* models. In addition, its effect on HBsAg level was compared to entecavir (ETV), and its co-treatment with ETV was performed to observe combination efficacy. After HPLC analysis of ziyuglycoside I and II, the effects of those compounds on HBsAg levels were measured.

Results: SOE, ziyuglycoside I, and II significantly reduced HBsAg excretion and the pgRNA levels in HepG2.2.15 cells. In particular, SOE treatment of 100 μg/ml showed an inhibitory effect on HBsAg excretion similar to the levels found with ETV treatment of the same concentration. Additional use of 125 μg/ml of SOE in combination with 80 μM of ETV inhibited HBsAg production up to the level that is diminished by 320 μM ETV treatment in HepG2.2.15 cells. Among SOE, ziyuglycoside I, II, and ETV, ziyuglycoside II had the lowest inhibitory concentration of 50% against genotype D, and it was more potent against HBV genotype C than D in down-regulating HBsAg secretion.

Conclusion: Taken together, SOE noticeably inhibited replication and antigen secretion of HBV, which was at least in part attributed to ziyuglycoside II, thus presenting the possibility of developing therapeutic candidates for the treatment of HBV-related diseases.

1. Introduction

Hepatitis B virus (HBV), a 3.2 kb DNA virus belonging to the hepatotropic *Hepadnaviridae* family [1,2], is an important pathogen that causes liver diseases such as viral hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [3,4]. To combat HBV infections, an effective and safe vaccine was developed in 1982 [5] and various antivirals were formally approved for CHB treatment including interferon (IFN) or nucleos(t)ide analogues such as lamivudine (LMV), telbivudine, adefovir dipivoxil (ADV), entecavir (ETV), and tenofovir (TDF) [6].

Despite continual progress in therapeutic strategies against HBV [7], the current antivirals suppress viral replication but are impossible to completely eradicate HBV [8]. The need for this type of long-term

* Corresponding author at: College of Pharmacy, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul, 02447, Republic of Korea.
** Corresponding author at: College of Korean Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea.
E-mail addresses: innks@khu.ac.kr (K.-S. Inn), komclive@khmc.or.kr (J.-H. Lee).
[1] Equal contributors.

https://doi.org/10.1016/j.eujim.2018.05.009
Received 6 March 2018; Received in revised form 17 May 2018; Accepted 18 May 2018

management unpredictably led to undesirable side effects and drug resistance. For example, nephrotoxicity was observed particularly in groups treated with ADV and TDF [9]; approximately 30–76% of patients treated with LMV and ADV showed a refractory response to the antivirals after a 5-year prescription [10].

HBV genotypes determine geographical distributions [1], response to antivirals, and disease progression [11]. Among them, genotype C, predominant in Southeast Asia, China and Korea, and genotype D, prevalent in Europe, Africa, Mediterranean countries and India, are closely associated with late HBeAg seroconversion, uncommon HBsAg seroclearance, low response to IFNs, and a high risk of developing HCC than patients with HBV genotypes A and B [11,12].

Sanguisorba officinalis (SO, Burnets in the West countries; Ziyu in Korea and Japan; Di-Yu in China), which belongs to the Rosaceae family, was introduced in Donguibogam where it was mainly used for the treatment of hematemesis and bloody diarrhea [13]. A recent report reviewed its various pharmacological effects against atherosclerosis, vascular diseases, Alzheimer diseases, bronchial asthma, and hair loss [14]. In particular, SO is a major herbal medicine of *Injinchunggan-tang* that has been prescribed to treat hepatitis B virus (HBV)-related liver diseases in Korea where the prevalence of CHB patients is relatively high. However, it is still unclear whether SO, ziyuglycoside I, and II have potent antiviral activities against HBV genotype C prevalent in Asian countries [11], and whether they could be alternative therapeutic candidates to overcome genotype-related vulnerability and weaknesses of modern antivirals.

In this study, we investigated whether 30% ethanol extract of SO (SOE) exhibits inhibitory effects against the production of pregenomic RNA (pgRNA) and antigens in HepG2 cells transfected with DNA genotype C as well as in HepG2.2.15 cells. None of the previous studies used the HBV genotype C in vitro model to identify the antiviral activities of SOE. In addition, its pharmacological activities were compared to a conventional antiviral such as ETV, and its antiviral parameters were observed when used in combination with ETV. Furthermore, ziyuglycoside I and II, which are active constituents of SOE, were treated to check their potential as inhibitors against HBV genotypes C and D.

2. Materials and methods

2.1. Plant material

Dried roots of the SO were purchased from Kyung Hee Herb Pharm (Wonju, Korea), a licensed herbal company, which was equipped with the Good Manufacturing Practice (GMP) facilities. The plant was obtained from Anhui province, China and the fresh roots of SO were authenticated at Dong-Eui Herbal Medicine Analysis Center Co. Ltd. (Busan, Korea). A voucher specimen, a representative sample of this plant, has been stored in the sample storage of Kyung Hee Herb Pham, Wonju, Korea for its identification and additive evidence (voucher number: SAN-1503).

2.2. Preparation of reagents

A total of 30 g of the SO plant material was immersed in 500 ml of 30% ethanol overnight at room temperature and then the supernatant extract obtained through the filtration process was collected. In clinical settings, herbal decoction is usually used as a water decoction extracted with hot water. However, for the purpose of this in vitro study using herbal extract, we selected 30% ethanol extract for higher efficacy without high risk in safety issues. In Korea, 30% ethanol solvent system is commonly recognized as nearly equivalent to water extraction and there are required to suggest more additional data in case of ethanol extraction (> 30%) due to the potential toxicity. Next, the SOE was freeze-dried using an Ilshin programmable freeze dryer apparatus (Ilshin Lab. Co., Ltd., Korea) for 72 h (freeze-dried powder mass = 4.33 g, yield = 14.43%). The freeze-dried extract was dissolved in dimethyl sulfoxide (DMSO) to prepare several stock solutions of 50 mg/ml, and these concentrated solutions were stored at 4 °C. Ziyuglycoside I and II (each 20 mg) with 98.0% purity were purchased from the WUHAN ChemFaces biochemical company (Hubei, China), and they were dissolved with DMSO to make a stock solution of 100 mM. ETV with 99.44% purity was bought from REYON pharmaceutical company (Chungbuk, Korea), and stored at room temperature.

2.3. Cells and virus

HepG2 cells (ATCC® HB-8065™, 15-year-old Caucasian male hepatoma derived cell line) and HepG2.2.15 cells (HepG2 cells transfected with HBV genotype D [15]) were maintained and cultured in a Dulbecco's Modified Eagle Medium (DMEM, HyClone™, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Equitech-bio. Inc., Cotton Gin Lane, USA) and 1% antibiotic penicillin (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 °C in a humidified atmosphere of 5% CO_2. In this study, the HBV genotype C (Hepatitis B virus isolate He74, complete genome) was used to transfect it into HepG2 cells. The virus was provided by the Liver Research Institute of Seoul National University (Seoul, Korea) and the virus stock (DNA concentration of 2275.8 ng/μl) was stored at −4 °C until needed.

2.4. HBV DNA genotype C transfection

HepG2 cells, an entirely differentiated human hepatoma cell line, were seeded at a density of 5×10^6cells in a 100 mm dish in DMEM media containing 10% FBS and 1% penicillin one day before transfection. Then, the DMEM media was freshly changed into 10 ml of reduced serum media (Opti-MEM®, Thermo Fisher Scientific Inc., USA) an hour before transfection. For transfection of the HBV DNA genotype C into HepG2 cells, 10 μg of the HBV DNA genotype C and 50 μl of Lipofectamine® 2000 (Thermo Fisher Scientific Inc., USA) were prepared. Lipofectamine® 2000 is a proprietary reagent for transfecting nucleic acids into a wide range of eukaryotic cells. This formulation and the HBV DNA genotype C were each diluted in 500 μl Opti-MEM® media, and then the diluted genotype C solution was added to diluted Lipofectamine® reagent drop by drop by gently vortexing. After 5 min of incubation at room temperature, we evenly treated 1 ml of the mixture of genotype C and lipofectamine in a drop-wise fashion over a 100 mm dish and incubated it overnight at 37 °C in a humidified atmosphere of 5% CO_2.

2.5. HPLC analysis

SOE was subjected to the high-performance liquid chromatography (HPLC) analysis with the standard products, ziyuglycoside I and II, in the Kyung Hee Drug Analysis Center, College of Pharmacy, Kyung Hee University (Seoul, Korea). The HPLC system consisted of a Waters model 515 HPLC pump, a model 717 plus autosampler and a model 2996 photodiodearray detector set to 203 nm (Waters, Milford, MA, USA). Separations were achieved at 40 °C using a Capcell Pak C18 (4.6 × 150 mm, I.D., 5 μm, Shisedo, Tokyo, Japan). The mobile phase used for the analysis consisted of solvent A (50 mM sodium phosphate) and solvent B (acetonitrile). The gradient elution was as follows: in 3 min 10% B; in 7 min from 10 to 90% B; in 5 min at 90% B; in 1 min from 90% to 10% B; and, in 9 min 10% B. The mobile phase was filtered through a 0.45 μm filter and delivered at a rate of 0.5 ml/min. Chromatographic data were collected and analyzed using Empower Chromatography data software (Waters, Version 5.0). The wavelength range of a photodiodearray detector was set up from 190 nm to 450 nm, and ziyuglycoside I and II chromatograms were measured at 203 nm for quantitation. The peak retention times of ziyuglycoside I and II were 11.5 min and 13.6 min, respectively.