RESEARCH ARTICLE

Comparison of the anti-H1N1 influenza virus effects of two *Scutellaria baicalensis Georgi* extracts

Xiwen Zhang*

College of Life Sciences, Northeast Agricultural University, Harbin, Heilongjiang, China.

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***Scutellaria baicalensis Georgi* (SBG) belongs to the commonly used traditional Chinese medicines, which has been used for more than 2,000 years in the history of China. In recent years, with the in-depth clinical research on the anti-influenza virus effect of SBG, it was found that the extract of SBG had an inhibitory effect on influenza A virus. The purpose of this study was to provide basic data and an experimental basis for the study of SBG against influenza A virus. Two kinds of extraction preparations were obtained by using liquid-liquid bi-directional extraction for refined preparation and ordinary decoction for crude preparation. The inhibitory effect on H1N1 influenza virus was detected by virus inhibition index and cell survival rate. The cell protective effects of the two preparations were detected by using methyl thiazolyl tetrazolium (MTT) and cloud point extraction (CPE) technologies to explore their mechanisms. The results showed that both extracts had good inhibitory effects on influenza A virus, in which the antiviral effects of the refined preparation were significantly stronger than that of the crude preparation, and this difference might be related to the different roles played by different components of *Cynodon grandiflorum* in exerting anti-influenza viral effects. The refined preparation contained a higher content of the active ingredient and therefore had stronger biological activity. The results of this study provided a reference for clinical rational drug use.**

Keywords: *Scutellaria baicalensis Georgi*; baicalin; liquid-liquid bi-directional extraction; ordinary decoction; influenza virus.

***Corresponding author:** Xiwen Zhang, College of Life Sciences, Northeast Agricultural University, Harbin 150000, Heilongjiang, China. Email: w2023ydnb@163.com.

Introduction

With the continuous development of modern medicine, the threat of viral diseases to human health has become increasingly serious. Under such circumstances, the search for natural drugs with antiviral effects has become a hot spot of research. Influenza (flu) is an acute infectious disease caused by influenza viruses, characterized by rapid transmission, high degree of contagiousness, and severe pathogenicity. The viruses are categorized into types A, B, and C and are prone to mutation, leading to differences in pathogenicity and antigenicity [1-3]. Seasonal and pandemic influenza are mainly caused by mutations in viral antigenicity with mutated hemagglutinin (HA) and neuraminidase (NA) causing small and large influenza, respectively [4]. The 2009 A(H1N1) virus, the most widespread class of viruses with significantly higher virulence and capacity, has triggered a global pandemic [5]. In order to cope with new variants of influenza viruses, the key lies in an in-depth understanding of the molecular mechanisms of influenza pathogenesis and the immune response of the body, and in the continuous search for new drugs to prevent and resist influenza virus infections [6]. SBG, as a

traditional Chinese herbal medicine, has a long history of medicinal use in China and has been widely used in the treatment of a variety of diseases [7, 8]. In recent years, it has been found that some of the active components in SBG have significant antiviral effects [9]. In the study of drugs, each drug has its unique efficacy and adverse effects due to the different active ingredients. The main components contained in SBG are flavonoids such as baicalin, quercetin, isoquercetin, *etc*., which have antiviral and anti-inflammatory and analgesic effects [10].

At present, the main extraction methods of SBG extract domestically and internationally are decoction method, reflux method, regulatory enzymatic method, semi-bionic extraction method, ultrasonic method, microwave method, and so on. These methods have some problems such as time-consuming, low efficiency, and high energy consumption, so they are difficult to meet the extraction needs of bioactive components with high efficiency, environmental protection, and energy saving. SBG liquid-liquid bi-directional extraction is an efficient, green, and energy-saving extraction technology. The principle of this method is that SBG raw materials and extractants are mixed, and the bioactive components are desorbed from the raw materials by means of oscillation and ultrasonic wave. Then, the stripping agent is used to separate the target component from the extractant. The method has the advantages of simple operation, high extraction efficiency, and environmental protection.

Although every influenza outbreak has corresponding new drugs to prevent or resist the spread of the disease, it is difficult for human to prevent because new variants of influenza virus may appear at any time. Therefore, a better understanding of the molecular mechanisms of influenza and the immune response of the body, and constantly looking for new drugs to prevent and resist influenza virus infection is the key to solve the re-outbreak of influenza. To obtain better anti-influenza virus effect, different methods of SBG extract should be examined, and the effective components of SBG need to be further explored. Therefore, in this study, SBG extract refined preparation and crude extract preparation were prepared, respectively, and their antiviral effects were compared. By comparing the two extraction methods, the optimal extraction process was determined to better study the pharmacodynamic mechanism and action of SBG, and to provide a theoretical basis for the development of new drugs for H1N1 influenza virus. This study provided a new way for the rational utilization of the resources of SBG, helped to promote the modernization process of traditional Chinese medicine, and provided strong support for the research in related fields.

Materials and methods

SBG sample preparation

SBG obtained from Anguo Shenhe Traditional Chinese Medicine Co., Ltd. (Anguo, Hebei, China) was placed in a 60℃ DHG-9075AE drying oven (Shanghai Jiecheng Experimental Instrument Co., Ltd., Shanghai, China) for 8 hours. After drying, SBG was ground into powder by using a TL150-Y crusher (Jiangsu Hengmin instrument Manufacturing Co., Ltd., Yancheng, Jiangsu, China), sifted through 40 mesh, bottled and sealed.

SBG crude extract preparation

Extraction was carried out using the common decoction method [11]. Briefly, 5 g of SBG powder was added to water and decocted for 1 h. The decoction was repeated three times with each time for 1 h and the first time using 10 times the amount of water and the second and third times using 8 times the amount of water. The decoctions were combined and filtered. The filtrate was heated to 80℃ and the pH was adjusted to 1.5-2 with 2 mol/L hydrochloric acid. The filtrate was kept warm for 1 h, and then stood at room temperature for 24 h. The supernatant was removed using SHB-111 vacuum pump (Zhengzhou Great Wall Science, Industry and Trade Co., Ltd., Zhengzhou, Henan, China) to recover the precipitation. The

precipitate was washed with water until the pH reaching 5.0, then washed with 70% ethanol to make the pH reaching 7.0, before dried at 60℃. The dried powder was collected and prepared with distilled water at a concentration of 10mg/mL.

SBG refined extract preparation

The SBG refined extract was prepared using liquid-liquid bi-directional extraction method [12]. After boiling 30 mL of phosphate buffer (pH 2.0), 5g of *Scutellaria baicalensis* powder was added and cooled to about 38℃ in LND-1006 liquid-liquid bi-directional extraction instrument (Shandong Luodani Analytical Instrument Co., Ltd., Zaozhuang, Shandong, China). Ethyl acetate was added from the upper end of the reflux tube in a funnel to the "b" bottle, while the "a" bottle was immersed in an oil bath at 38℃. The "b" bottle was heated and stirred to make ethyl acetate forming reflux. The ethyl acetate solution was collected after reflux extraction for 4 hours, and the ethyl acetate was recovered on the RMI-1005 rotary evaporation instrument (Zhengzhou Keda Machinery Equipment Co., Ltd., Zhengzhou, Henan, China) to get the medicinal extract. The distilled water was added to 500 mg accurately weighed SBG extract to prepare 50 mL of solution.

High-performance liquid chromatography (HPLC) analysis

100 mL of 70% ethanol was added to 1 g of each sample extract and dissolved for 30 mins in a water bath (Beijing Medical Equipment Factory Co., Ltd., Beijing, China) and then filtered. 5.0 mL of filtrate was mixed with 70% ethanol to reach the volume of 50 mL. Further, 1.0 mL of the filtrate was mixed with 20 mL of 0.2 mol/L hydrochloric acid and 70% ethanol to the volume of 25 mL. 5 µL of sample was applied to determine the absorbance at 335 nm wavelength.

Cytotoxicity assay

A549 cells obtained from the School of Basic Medicine, Peking Union Medical University, Beijing, China were resuscitated and inoculated in aseptic petri dishes with the cell concentration of 1.5×10^8 cells/L. 100 µL of cells were inoculated to each well of a 96-well plate and incubated in a WMK-02 Carbon Dioxide Incubator (Guangzhou Kangheng instrument Co., Ltd., Guangzhou, Guangdong, China) at 35℃ until the bottom of each well covered by monolayer cells [13]. The two different extracts each at 10 mg/mL were diluted 10 times, and 100 µL of extract was added to each well on 96-well plate with triplicates for each concentration. The cells were then cultured in carbon dioxide incubator at 35℃ for 48 hours before the cell morphology was observed. The maximum non-toxic concentration of the drug (TD_0) was determined using HBS-1096A enzyme labeling instrument (Nanjing Detie Experimental Equipment Co., Ltd., Nanjing, Jiangsu, China). The OD values of crude extraction, refined extraction, and blank control were measured, and the cell survival rate was calculated below.

Cell viability (%) = (OD value of crude or refined group/OD value of control group) × 100%

Determination of half-cell toxic concentration of H1N1 influenza virus $TCID_{50}$

The H1N1 influenza virus obtained from Beijing Institute of Traditional Chinese Medicine, Chinese Academy of Traditional Chinese Medicine, Beijing, China was cultured in a serum-free medium. The crude and refined SBG extracts were mixed with 200 $TCID_{50}$ H1N1 virus solution to the concentrations of TD_0, ½ TD_0, and ¼ TD_0. The virus control group used cell culture medium (1% penicillin/streptomycin mixture + 10% fetal bovine serum McCoy's 5A medium) instead of SBG extract. The reaction was incubated at 35℃ for 2 hours before transferring to the cell culture medium and continuing to culture for 48 hours. The micromorphology of the cells was observed after incubation, and the number of holes and the degree of pathological changes were recorded. When the cell control group was close to the normal morphology, the holes with the disease rate ≥ 50% of the infected cells were used as disease holes, and the $TCID_{50}$ infection amount of the virus and the virus inhibition index of the

two extracts were calculated.

Virus inhibition index = virus control log$TCID_{50}$ - formulation log$TCID_{50}$

Cell mortality (%) = (cell control-experimental group)/cell control

$TCID_{50}$ = 50% concentration of cell death

Antiviral effects

(1) Microscopic observations

The virus dilution of 100 $TCID_{50}$ was inoculated into the 96-well plate of monolayer cells with 100 µL per well. After culture in the incubator for 2 hours, 10 µL of diluted crude extract and 100 µL refined extract were added in turn with the normal cell control group and virus control group. The cytopathic effect was observed after 24 hours by using Olympus GX53 inverted microscope (Olympus Corporation, Hachiko City, Tokyo, Japan) and verified by repeating experiment for 3 times. The degrees of cell lesion were defined as "+" (0 - 25%), "++" (25 - 50%), "+++" (50 - 75%), "++++" (75 - 100%). The experimental results were recorded when the virus control group cell lesions were ++++.

(2) MTT assay

100 µL of 100 $TCID_{50}$ of virus dilution was added to each well of monolayer cells and incubated in the incubator for 2 h before aspirating the influenza virus. The crude and fine SBG extracts diluted in pairs of times below the maximum nontoxic concentration were added with the cell and virus control groups being set up. 10 µL of MTT was added to each well after 48 h and the OD value at the wavelength of 570 nm was detected. The lesion inhibition rate (ER) was calculated by the following formula:

Lesion inhibition rate (ER) = (average OD value of drug - average OD value of virus control group) / (average OD value of cell control - average OD value of virus control group) × 100%

Statistical analysis

SPSS 25.0 statistical software (IBM, Armonk, New York, USA) was employed in this study. Mean ± standard deviation (x ± s) was used to express normality. Chi-square and one-way ANOVA were used to compare the differences between the experimental groups. Two-by-two comparisons between groups were made using the LSD method. The P value less than 0.05 indicated the significant difference.

Results

HPLC detection of two extract samples

The HPLC results showed the types of chemical components in the SBG extracts prepared through the different extraction methods were basically the same (Figure 1). Both extracts were all out of the column within 14 mins with the peaks of baicalin shown at 6 mins.

Toxic effects of SBG extracts on A549 cells

The A549 cells became rounded, partially broken, and detached from the wall of culture wells when treated with two SBG extract stock solutions. When treated cells with 100-fold dilution, the cell morphology changed greatly, and there was a certain gap with the normal cells. The inhibition rate of the cells in the refined group was 58.26% and that of the cells in the crude extract group was 59.63%. However, starting from a dilution of 1,000-fold, the cell inhibition rate of the refined group was 96.64%, the cell inhibition rate of the crude extract group was 97.47%. There was almost no gap between the normal cells (Table 1). Because the cell inhibition rate changed so suddenly in 10-times dilutions, double dilutions were applied. The results showed that the A549 cells began to show lesions when the refined group was diluted up to 800-fold and the crude extract group was diluted up to 400-fold (Table 2). Therefore, the SBG concentrations of refined extract being diluted up to 1,000-fold and the crude extract being diluted up to 800-fold were set as the maximum non-toxic concentrations. The TD_0 numbers of the refined and crude extract groups were 1 mg/mL and 1.25 mg/mL, respectively.

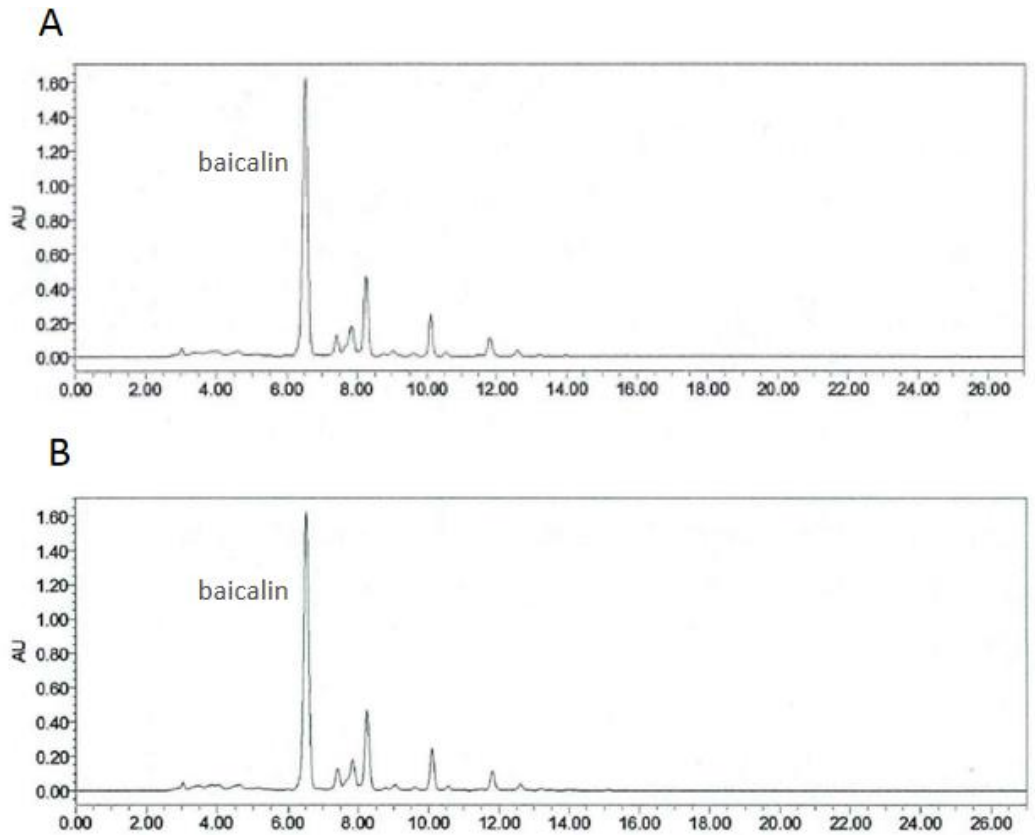

Figure 1. HPLC chromatogram of baicalin. **A.** crude extract preparation. **B.** refined extract preparation.

Table 1. Toxic effects of two extract preparations on A549 cells (10-fold dilution).

Dilution ratio	Cellular pathology		Cell survival rate (%)	
	Refined extract group	Crude extract group	Refined extract group	Crude extract group
1	++++	+++	13.36 ± 1.32	26.17 ± 1.56
10	+++	+++	33.32 ± 3.21	30.33 ± 2.62
100	++	++	58.26 ± 3.36	59.63 ± 3.48
10,00	-	-	96.64 ± 5.64	97.47 ± 4.47
10,000	-	-	97.57 ± 5.12	98.45 ± 4.36
100,000	-	-	98.21 ± 4.38	98.83 ± 5.22

Notes: cytopathic (death) degree: "-": 0%. "+": 0-25%. "++": 25-50%. "+++": 50-75%. "++++": 75-100%.

Table 2. Toxic effects of two extract preparations on A459 cells (double dilution).

Dilution ratio	Cellular pathology		Cell survival rate (%)	
	Refined extract group	Crude extract group	Refined extract group	Crude extract group
100	++	++	57.62 ± 2.38	58.97 ± 3.31
200	++	++	68.22 ± 4.61	73.57 ± 4.12
400	+	+	81.34 ± 2.87	87.35 ± 3.28
800	+	-	89.63 ± 4.32	94.33 ± 2.46
1,600	-	-	97.12 ± 3.66	97.67 ± 3.48
3,200	-	-	97.24 ± 3.91	97.87 ± 4.53

Notes: cytopathic (death) degree: "-": 0%. "+": 0-25%. "++": 25-50%. "+++": 50-75%. "++++": 75-100%.

Table 3. Effect of two groups of SBG extracts on H1N1 virus virulence *in vitro* (n=4).

Group	logTCID$_{50}$			Virus Suppression Index		
	TD_0	½ TD_0	¼ TD_0	TD_0	½ TD_0	¼ TD_0
Refined extract group	3.37 ± 0.12	4.11 ± 0.23	4.44 ± 0.32	2.29 ± 0.33	1.55 ± 0.42	1.22 ± 0.45
Crude extract group	3.86 ± 0.37	4.65 ± 0.34	5.22 ± 0.41	1.8 ± 0.51	1.01 ± 0.28	0.44 ± 0.18
Virus control		5.66			-	

Table 4. Effect of two SBG extract groups on cytopathic effects (CPE) and lesion inhibition rate (ER) of H1NI virus (n=4).

Drug concentration	CPE		ER (%)	
	Refined extract group	Crude extract group	Refined extract group	Crude extract group
TD_0	+	+	93.88 ± 4.21	82.24 ± 3.72
½ TD_0	+	++	75.64 ± 4.26	66.32 ± 3.81
¼ TD_O	++	++	64.03 ± 1.18	60.58 ± 2.86

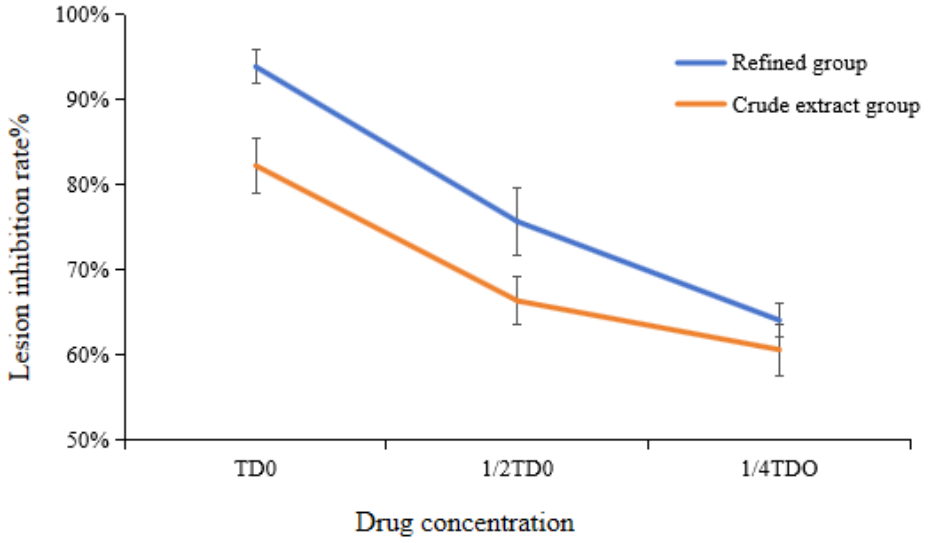

Figure 2. Comparison of antiviral efficacy of the two SBG extracts.

Effect of SBG on virulence of H1N1 virus

The TCID$_{50}$ of H1N1 virus was calculated by using Reed-Muench method and it was found that with the increase of virus solution, both groups of SBG extracts showed an increasing trend in titer of the virus, and when the concentration of the refined extract group was TD_0, it showed a significant inactivation effect with an inhibitory viral index of 2.29, while the crude extract group had an nonsignificant inhibitory viral index of 1.8 at TD_0. Therefore, the refined extract group was more effective in inactivating the virus. (Table 3).

Effect of SBG on the cytopathic effects of viruses

When the concentrations of the two SBG extract groups were TD_0, the lesion inhibition rate was 93.88% for the refined extract group and 82.24% for the crude extract group. Both groups showed a decrease in viral inhibition as the concentration of the extract decreased, while the degree of fragmentation, increasing refractive index, and roundedness of the cells became more and more pronounced in the lesions (Table 4). The antiviral potency of the SBG refined extract group was significantly different from that of the crude

extract group, and the antiviral difference between the two groups was gradually obvious with the increase of concentration (Figure 2). The results showed that SBG refined extraction method had better antiviral effect against H1N1 virus.

Discussion

Influenza viruses are the main pathogens that cause pandemic influenza with influenza A (H1N1) virus being particularly prominent. The genes of influenza A virus are prone to recombination, leading to antigenic drift and conversion, generating new subtypes of viruses to attack the organism and causing the organism to lose immunity to the virus [14-16]. Therefore, it has become an urgent task to find new anti-influenza virus drugs to address the diversity of influenza virus subtypes. Single and compound preparations of Chinese medicines such as SBG, *Coptis chinensis Franch*, and *Lonicera japonica Thunb* have demonstrated unrivaled advantages against influenza viruses [17]. The antiviral activity of SBG is mainly attributed to its rich flavonoids, especially baicalin and baicalein [18, 19]. These compounds have significant antiviral effects and can effectively inhibit viral replication and infection [20]. The antiviral properties of SGB have been known for a long time, and its extract isobaicalein-8-methyl ether significantly inhibited influenza virus activity [21]. In addition, baicalein is a potent inhibitor of murine leukemia virus (MLV) and human immunodeficiency virus (HIV) reverse transcriptase with 2 μg/mL of baicalein inhibiting MLV and HIV reverse transcriptase activity by more than 90% [22]. It was also found that baicalein was not cytotoxic to CEM, an *in vitro* cultured T cell line, at certain concentrations, but exhibited significant cytotoxicity to HIV-infected CEM cells, resulting in cellular DNA breaks [23, 24]. The anti-HIV effect of baicalin was particularly pronounced in CEM-HIV cells with high volume release of HIV virus, which might be related to its selective induction of apoptosis in CEM-HIV cells caused by cells with high viral release [25].

The antiviral active components in SBG are the key to its antiviral effects. To further understand the specific mechanism of the antiviral action of SBG, the common decoction extraction method and liquid-liquid bidirectional extraction method were used in this study for the extraction of SGB. The HPLC profiles of the different SGB preparations revealed that the types of chemical components from the two different SBG extracts were basically the same. In the drug concentration TD assay, the concentration of baicalin was similar in both SBG extracts with a TD_0 of 1 mg/mL in the refined extract group and 1.25 mg/mL in the crude extract group. When the concentration of the SBG refined extract group was TD_0, the viral inhibition index was greater than 2, showing significant direct viral inactivation, whereas for the 3 middle concentrations of the SGB crude extract group, the viral inhibition indices were less than 2, indicating that direct viral inactivation was not significant. When the concentrations of both extract groups were TD_0, the lesion inhibition rates were 93.88% and 82.24% for the refined and crude extract groups. Both groups showed a decrease in viral inhibition as the concentration of the extracts decreased. The antiviral potency of the two extracts was significantly different and the antiviral difference between the two groups was gradually obvious with increasing concentration. Both SBG refined and crude extracts possessed antiviral effects, but the antiviral effect of the SBG refined extract was more pronounced, and this difference might be related to the different contents of the active ingredients of the extract. In future studies, the specific mechanism of the antiviral effects of SBG should be further explored, and other antiviral active components in SBG also need to be studied in depth to provide new directions for the development of novel antiviral drugs.

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