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<Short Communication>

Antibacterial and therapeutic effects of a combination of *Sophora flavescens* and *Glycyrrhiza uralensis* Fischer ethanol extracts on mice infected with *Streptococcus pyogenes*

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Abstract : This study evaluated the antibacterial effects of a mixture of *Sophorae radix* and *Glycyrrhiza uralensis* Fischer (1 : 1) ethanol extracts (SGE) on mice infected with *Streptococcus* (*S.*) *pyogenes*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration of SGE necessary for antibacterial effects against *S. pyogenes* were 20 μg/mL. Based on the time-kill curves for *S. pyogenes*, SGE was effective at 4× MIC after 16 h. On Day 12 after challenge, the survival rate of mice treated with 2.0 mg/kg SGE was 60%. In conclusion, SGE had potent *in vitro* and *in vivo* antibacterial activities against *S. pyogenes*.

Keywords : crude extracts, medicinal plants, mice, *Streptococcus pyogenes*, treatment efficacy

Streptococcus (*S.*) *pyogenes*, a group A streptococcus, is an aerobic, spherical, gram-positive extracellular bacterium that is associated with a variety of mucosal and invasive human infections [3]. These bacteria cause various diseases ranging from mild and quite frequent non-invasive infections of the upper respiratory tract and skin to severe invasive infections that include necrotizing fasciitis and streptococcal toxic shock syndrome [4].

The most important modes of *S. pyogenes* transmission are via respiratory droplets, hand contact with nasal discharge and skin contact with impetigo lesions [1]. In addition, *S. pyogenes* can be spread to cattle and then back to humans through raw milk as well as contaminated food sources such as salads, milk and eggs [7].

In a previous study [16] that analyzed for antimicrobial susceptibility against *S. pyogenes* isolates in scarlet fever patients between 2003 and 2011 in China, 100, 97.0, and 89.4% of the 74 *S. pyogenes* isolates were found to be resistant to erythromycin, clindamycin and tetracycline, respectively. The emergence of antibiotic resistance has led to the search for new, safe and effective antimicrobial agents from alternative natural resources. Medicinal herbs as alternatives to antibiotics are attracting considerable attention by many researchers, and many such herbs have a long history of medicinal use in Asia [14]. Many of these herbs are often used in combination to increase their effects. These herbs containing bioactive components have many potential clinical and therapeutic applications in modern medical care [11]. In addition, antimicrobial agents can be derived from herbs, and more than 1,340 plants have been reported to have antimicrobial effects [15].

Sophora (*S.*) *flavescens* which has antibacterial, antiinflammatory, antipyretic, antiulcerative and antineoplastic effects has been used traditionally as medicinal herbs in the treatment of jaundice, leucorrhea, carbuncles, pyogenic infections of the skin, scabies, enteritis, and dysentery [8, 9]. *Glycyrrhiza* (*G.*) *uralensis* Fischer is one of the most generally used herbal medicines in the world and has been proved to possess antiinflammatory, liver protection, antibacterial, and anticancer activity, which are attributed to the contained triterpenoid saponins and flavonoids [5].

This study evaluated the antibacterial activity of a combination of ethanol extracts from the medicinal herbs, *S. flavescens* and *G. uralensis* Fischer, against *S. pyogenes*. In addition, a preparation of the herbs was fed to mice infected with *S. pyogenes* in order to determine its therapeutic poten-

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tial *in vivo*.

S. flavescens and *G. uralensis* Fischer purchased from the Korea National Animal Bio Resources Bank (Jinju, Korea) were isolated from plant materials as described previously [12]. Briefly, each medicinal plant was air-dried in a dark room and ground to a powder. Approximately 50 g of the powdered materials was soaked in 150 mL of ethanol for 24 h at room temperature under mantle-reflux. The solvent was removed under reduced pressure in a rotary evaporator (EYELA; Tokyo Rikakikai, Japan) at 34°C. The extracts were first filtered using Whatman No.1 filter-paper, and the filtrate was concentrated using a vacuum rotary evaporator (Iwai, Japan), followed by freezing of the dried powder.

S. pyogenes (KCTC 3208) was obtained from the Korean Collection for Type Culture (Daejeon, Korea). The bacterium was cultured in brain-heart infusion (BHI) broth (Difco, USA) supplemented with 2% supplement B (Difco) under the condition of 37°C with 5% CO_2 for 24 h.

The minimum inhibitory concentration (MIC) of *S. flavescens* (SFE) and *G. uralensis* Fischer (GUE) extracts was estimated using the broth dilution method in BHI broth. Briefly, SFE and GUE powders were diluted in ethanol and serial dilutions were prepared. A 1 mL aliquot of bacterial strain culture, containing 1×10^6 colony forming unit (CFU)/mL, was added to tubes containing BHI broth supplemented with SFE and GUE at concentrations ranging from 10 to 100 μg/mL. Controls without the herbal extract were prepared. Erythromycin (Sigma-Aldrich Korea, Korea) was used as a reference antibiotic. After incubation for 24 h at 37°C under agitation, the MICs were determined as the lowest concentration of SFE and GUE inhibiting visible bacterial growth. According to the method of the previous study [10], to determine the minimum bactericidal (MBC), 10 μL of bacterial inoculum was taken aseptically from the tubes that had not presented visible turbidity and was plated onto sheep blood agar. The MBCs were considered as the lower concentration of SFE and GUE that allowed less than 0.1% of the original inoculum treated with the herbal extract to grow on the surface of the sheep blood agar. The MIC and MBC values were obtained from three independent experiments. Based on the MBC of SFE and GUE, a combination of SFE and GUE was prepared in a ratio of 1 : 1, and designated as SGE. According to the above method, the MIC and MBC of SGE were determined.

To analyze the bactericidal effect of SGE, 1 mL of the bacterial diluents (1×10^6 CFU/mL) was added to BHI broth containing different concentrations of SGE (0, 15, 30, 60 and 120 μg/mL). The mixture was incubated at 37°C for 24 h and the number of *S. pyogenes* cells was observed 0, 4, 8, 16, and 24 h post-incubation. At each observation point post-incubation, 1 mL of the culture medium was sampled and diluted. After dilution, 100 μL of each diluent was plated onto BHI agar and cultured for 24 h at 37°C with 5% CO_2 to determine the number of CFUs.

For the mouse challenge test, forty female BALB/c mice (5-week old) purchased from Samtako Bio Korea (Osan, Korea) were acclimated for 1 week, and anesthetized and inoculated by intraperitoneal injection with 0.1 mL of the bacterial suspension (1×10^8 CFU/mL). After inoculation, mice were randomly assigned to control and experimental groups, and divided into four groups of ten animals each. Mice in control were administered with normal saline, and animals in group A, B, and C were administered with SGE at doses of 0.5, 1.0, and 2.0 mg/kg body weight, respectively. All drug-treated groups received daily oral treatment of medications for 12 days. The survival rates of each group were checked and recorded every day before administration of the drug. In addition, liver and kidney markers were measured to identify the toxicity of SGE after the treatment period. All animal experiments were conducted under ethics approval from the Gyeongsang National University Animal Ethics Committee (GNU-140122-M0015) in accordance with the guidelines of the Korean Council on Animal Care.

Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS (ver. 15.0, SPSS; USA), including the analysis of variance and Student's t test. A value of $p < 0.05$ was considered statistically significant.

In the present study, the MIC and MBC of SFE was both 25 μg/mL and those of GUE were 20 and 25 μg/mL, respectively. In addition, the MIC and MBC of SGE were 15 and 20 μg/mL, respectively. On the other hand, the MIC and MBC of erythromycin were both 0.125 μg/mL (Table 1).

In time-kill curves of *S. pyogenes* after treatment, SGE exhibited killing effect at 4× MIC (60 μg/mL) and 8× MIC (120 μg/mL) after 16 and 8h, respectively, while the concentration at 2× MIC (30 μg/mL) and 1× MIC (15 μg/mL) inhibited growth of *S. pyogenes* throughout the incubation time. At 4 h post-incubation, the growth of *S. pyogenes* treated with SGE was significantly inhibited compared to that of the control (MIC, $p < 0.05$; 2× MIC, 4× MIC and 8× MIC, $p < 0.001$) (Fig. 1).

For the therapeutic effect of SGE on mice infected with *S. pyogenes*, mice in the control group and in group A started to die on day 2 after inoculation with *S. pyogenes*, and the survival rate dropped to 0% in the control group after day 6 and

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of medicinal plant extracts against *Streptococcus* (*S.*) *pyogenes*

Compounds	MIC (μg/mL)	MBC (μg/mL)
SFE	25	25
GUE	20	25
SGE	15	20
Erythromycin	0.125	0.125

SFE: *Sophora flavescens* (*S. flavescens*) ethanol extract, GUE: *Glycyrrhiza* (*G.*) *uralensis* Fischer ethanol extract, SGE: a combination of *S. flavescens* and *G. uralensis* Fischer extract, prepared in a ratio of 1 : 1 (*S. flavescens* : *G. uralensis* extract powder, w/w).

Table 2. Effect of SGE on murine liver and kidney functions after treatment for 12 days

Group	Liver function (U/L)		Kidney function (mg/dL)	
	ALT	AST	Creatinine	BUN
Control	62.4 ± 4.26	78.5 ± 4.35	0.43 ± 0.02	21.5 ± 2.14
group A	63.5 ± 3.89	78.6 ± 4.52	0.42 ± 0.03	21.2 ± 2.21
group B	64.1 ± 4.57	77.7 ± 4.41	0.43 ± 0.02	22.8 ± 2.39
group C	63.3 ± 4.64	77.9 ± 5.03	0.42 ± 0.03	22.9 ± 2.51

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BUN: blood urea nitrogen. Values represent the mean ± SD of three independent experiments. Control group: treated with phosphate buffer solution (PBS), group A: treated with 0.5 mg/kg SGE, group B: treated with 1.0 mg/kg SGE, group C: treated with 2.0 mg/kg SGE.

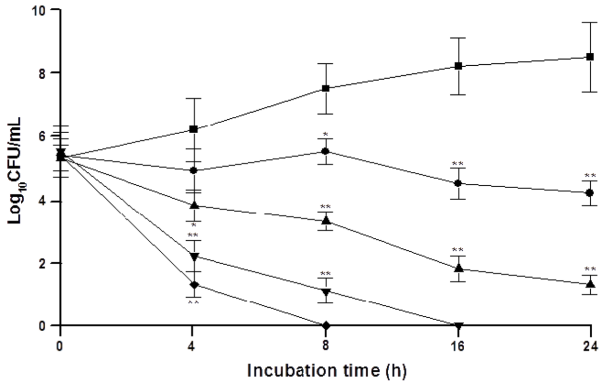

Fig. 1. The bactericidal activity of SGE against *pyogenes*. Different concentrations of SGE (0 (■), 15 (●), 30 (▲), 60 (▼) and 120 (◆) μg/mL) were diluted in PBS and incubated with *S. pyogenes* for 1, 4, 8, 16 and 24 h. Bacterial viability was monitored by measuring CFUs on culture plates, and the rate of bacterial viability was compared to the zero time point in the untreated PBS (control). The data represent the mean ± SD of triplicate experiments.

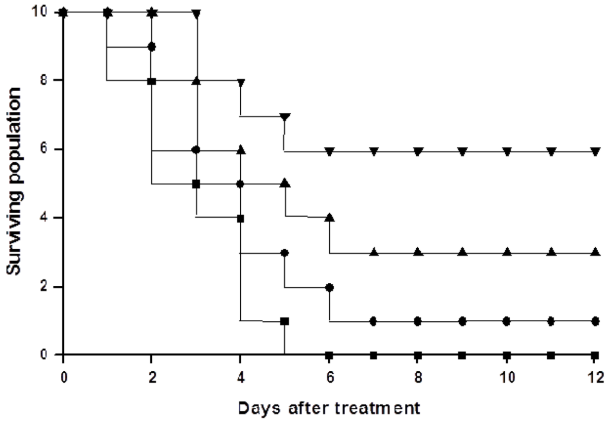

Fig. 2. Mortality rate of mice infected with *S. pyogenes* after treatment with SGE of different concentrations for 12 days. ■, control group treated with phosphate buffer solution (PBS) (n = 10); ●, group A treated with 0.5 mg/kg SGE (n = 10); ▲, group B treated with 1.0 mg/kg SGE (n = 10); ▼, group C treated with 2.0 mg/kg SGE (n = 10).

10% in group A after day 7. The survival rate of group B and C remained at 30% after day 7 and 60% after day 6, respectively (Fig. 2). Based on blood biochemical analysis results in this study, all parameters (alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN)) in the groups treated with SGE showed no significant differences compared to those of the control group (Table 2).

In a previous study, an ethanol extract of *Rhodomyrtus tomentosa* displayed potential antibacterial activity with the MIC and MBC ranged from 3.91 to 62.5 μg/mL against 47 clinical isolates of *S. pyogenes* [10]. Considering the above result, the antibacterial activity of SGE was similar to that of the ethanol extracts of *Rhodomyrtus tomentosa*.

In a previous study, a *Rhodomyrtus tomentosa* extract completely killed *S. pyogenes* at 8× MIC (3.12~12.48 μg/mL) 16 h post-incubation, while doses at 4× MIC and 2× MIC hindered growth of the bacterium [10]. Compared with the above results, the time-killing effect of SGE was higher than that of the *Rhodomyrtus tomentosa* extract.

The survival rate of mice in a previous study was 60% on day 7 after inoculation with *S. pyogenes* and simultaneous treatment with Hainosankyuto (Platycodon Root 4.0 g, Glycyrrhiza 3.0 g, Immature orange 3.0 g, Peony Root 3.0 g, Jujube 3.0 g, Ginger 1.0 g) 10 mg/10 g body weight for 4 consecutive days [13]. Considering the dose, time and period of administration, the therapeutic effect of SGE on mice infected with *S. pyogenes* might be stronger than that of Hainosankyuto.

S. flavescens contains a variety of biomaterials such as leachianone A, trifolirhizin, ketoconazole and sophoraflavanone G. Among them, sophoraflavanone G has potential antimicrobial activity against pathogenic bacteria [2]. In addition, *G. uralensis* has been proven to possess various bioactive compounds of which glycyrrhizin A and isoflavonoids exhibited potent antibacterial activity against pathogenic bacteria [6]. Thus, compounds such as sophoraflavanone G, glycyrrhizin A and isoflavonoids in SGE might display the synergistic antibacterial activity against *S. pyogenes* through interaction in our study.

With the results of *in vitro* and *in vivo* studies, SGE used in the present study displayed potent antibacterial and therapeu-

tic effects against *S. pyogenes* with no alteration to liver and kidney functions of mice after treatment for 12 days.

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