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Evaluation of Antibacterial Effects of a Combination of *Coptidis Rhizoma, Lonicerae Flos, Paeonia Japonica* Extracts, and Dioctahedral Smectite Against *Salmonella Typhimurium* in Murine Salmonellosis

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ABSTRACT - The present study was undertaken to estimate the antibacterial effect of a combination of *C. rhizoma, L. Flos, and P. japonica* (1:1:1) extracts (CLP1000) and a combination of the herbal extract mixture and dioctahedral smectite (CLPS1000) against murine salmonellosis. At the concentration of CLP1000 and CLPS1000 0.5 mg/ml, the antibacterial effect was not showed on *Salmonella typhimurium (S. typhimurium)*. On the other hand, the antibacterial effect against S. typhimurium was observed at the concentration of CLP1000 and CLPS1000 1.0 mg/ml. Oral administration of Smectite, CLP1000, and CLPS1000 at the dose of 10 mg/ml showed a therapeutic effect for *S. typhimurium* infected BALB/c mice. The mortality of Smectite, CLP1000 and CLPS1000-treated mice was 90%, 90%, and 70% at 12 days, respectively, while that of untreated mice was 100% at 9 days after a lethal dose of *S. typhimurium* infection. The results of our study strongly indicate that CLPS1000 has potential as an effective of salmonellosis.

Key words : *Coptidis rhizoma, Lonicerae Flos, Paeonia japonica*, dioctahedral smectite, *Salmonella typhimurium*

Salmonella extensively causes various disease syndromes, such as self-limiting enteritis, fatal infection in animals, food-borne infection, and typhoid fever in humans[1-4].

Generally, antimicrobial agents are used both therapeutically and prophylactically in salmonellosis. However, the increased resistance to these drugs is an unavoidably side effect of antibiotic use, and recent studies have shown a rapid increase in the prevalence of antibiotic resistant Salmonella[5-7].

To solve the problem of antibiotic resistance, medicinal herbs and minerals products are attracting considerable attention by many researchers. Many of medicinal herbs are often used in combination to increase their effects. As these herbs contain bioactive components, they have been used many potential clinical and therapeutic applications[8,9].

Coptidis rhizoma (*C. rhizoma*) has been used in oriental medicine as an antibacterial and anti-inflammatory agent[10,11]. The extract of *C. rhizoma* contains a high level of berberine and antibacterial activities in a variety of pathogenic microorganisms, including Salmonella, Pneumococcus, Mycobacterium tuberculosis, Staphylococcus[12,13]. *Lonicerae Flos* (*L. Flos*) is a widely used herb prescribed in many Korean formulas. It has antibacterial activity, antipyretic, detoxicant, and anti-inflammatory actions[14-17]. The aqueous extract of *Paeonia japonica* (*P. japonica*) has been used in oriental medicine to treat various illnesses because of possession various pharmacological properties such as sedative, analgesic, anti-inflammatory, antimicrobial, immunoaugmentative and anti-stress action[18-23].

Medicinal and therapeutic use of mineral products has impacted human health for thousands of years. The intentional consumption of clay materials by humans and animals is known as geophagy, a complex behavior, cosmetics, dietary or nutritional needs and medicinal benefits[24-26]. Some of clay mineral products have been reported to have anti-diarrheal and antibacterial activity[27-29].

Although previous studies had been investigated antibacterial and antidiarrheal effects for each of Korean traditional herbs and clay minerals, there is little study to investigate

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for the synergistic effect of the combination.

The present study evaluated the therapeutic potentials of the combination of dioctahedral smectite (Smectite) and methanol extract from the medicinal herbs, *C. rhizoma, L. Flos,* and *P. japonica* for their antibiotic properties against *Salmonella typhimurium* (*S. typhimurium*) *in vitro* and *in vivo*.

Materials and Methods

Preparation of a combination

C. rhizoma, L. Flos, and *P. japonica* were purchased from the Korea National Animal Bio Resource Bank (Gyeongnam, Korea). The combination of Korean traditional herbs was referred to Kim[30]. *C. rhizoma, L. Flos,* and *P. japonica* were air-dried in a dark room and ground to a powder. Approximately 100 g of the combination of *C. rhizoma, L. Flos,* and *P. japonica* (1:1:1) powder was soaked in 400 ml of methanol for 24 h under mantle-reflux. The solvent was removed under reduced pressure in a rotary evaporator (N-1000 S, EYELA, Japan). The extracts were filtered using Whatman No.1 filter paper, and the filtrates were evaporated to dryness in a steady air current. The extracts and the combination of herbal extracts and smectite were designated CLP1000 and CLPS1000, respectively. The extracts and the combination were dissolved in distilled water, adjusted to 100 mg/ml final concentration, and sterilized by passage through a Corning syringe filter (0.20 μm, Japan), respectively[31].

Bacterial culture and media

Salmonella enterica serovar Typhimurium (*S. typhimurium*) ATCC 14028 cells were maintained as frozen glycerol stock and cultured in Luria-Bertani (LB) broth or LB broth containing 1.5% agar. Bacteria were grown at 37°C with vigorous shaking to a stationary phase in LB broth.

Determination of antibacterial activity

Bacteria were diluted with phosphate-buffered saline (PBS) solution (pH 7.4) to 2×10^4 CFU/ml, added to different concentrations (0.5, 1.0 mg/ml) of CLP1000 and CLPS1000, respectively, and incubated at 37°C for 0, 2, 4, and 8h. After incubation and proper dilution, 100 ml of each solution was plated onto LB agar to assess bacterial colony forming units (CFUs).

Smectite, CLP1000, and CLPS1000 treatment for murine salmonellosis

Specific pathogen free (SPF) female BALB/c mice (Orient Bio, Seoul, Korea) aged 6-8 weeks, weighing 27.1 ± 3.8 g each, were used in this study. All mice were kept at 23 ± 1°C with a 12-h light/dark cycle. They had free access to water and diet and were acclimatized for at least 2 weeks before starting the experiments. Four groups of 10 mice each were used for bacterial infection. Mice were infected intraperitoneally with 2×10^4 CFUs of *S. typhimurium*[2]. After bacterial infection, three groups of mice were orally treated with 0.1 ml of sterile PBS, Smectite (10 mg/ml), CLP1000 (10 mg/ml), and CLPS1000 (10 mg/ml) every 24 h during 12 days, respectively. Infected mice were examined for the viability every 24 h. All procedures described were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University (GNU-LA-15).

Statistical analyses

The data were analyzed by a one-way analysis of variance (ANOVA), followed by Student's *t*-test. The results are expressed as mean ± SD. A mean difference was significant at the 0.05 level.

Results and Discussion

Determination of antibacterial activity

Antibacterial effect of CLP1000 and CLPS1000 against Salmonella enteric serovar Typhimurium (*S. typhimurium*) is presented in Fig. 1. At 2 h after incubation, inhibition of *S. typhimurium* growth in the all treated groups was significantly lower than that of control (no treatment) ($p < 0.05$). At 4 h and 8 h after incubation, inhibition of *S. typhimurium* growth in the all treated groups was significantly lower than that of control (no treatment) ($p < 0.001$), but the number of bacterial cells in the CLP100 and CLPS1000 group treated with 0.5 mg/ml was increased

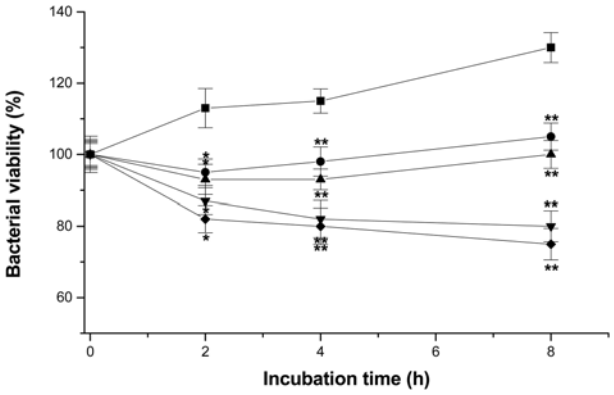

Fig. 1. Antibacterial effect of a combination of *C. rhizoma, L. Flos,* and *P. japonica* extracts (CLP1000) and a combination of the extract mixture and dioctahedral smectite (CLPS1000) against *S. typhimurium*. CLP1000 diluted with PBS was used at the concentration with 0 (■), 0.5 (●) and 1.0 (▼) mg/ml, and CLPS1000 was used at the dose of 0.5 (▲) and 1.0 (◆) mg/ml. Bacterial viability was measured based on CFUs on culture plates for three independent experiments. *Significantly different from the control ($p < 0.05$). **Significantly different from the control ($p < 0.001$).

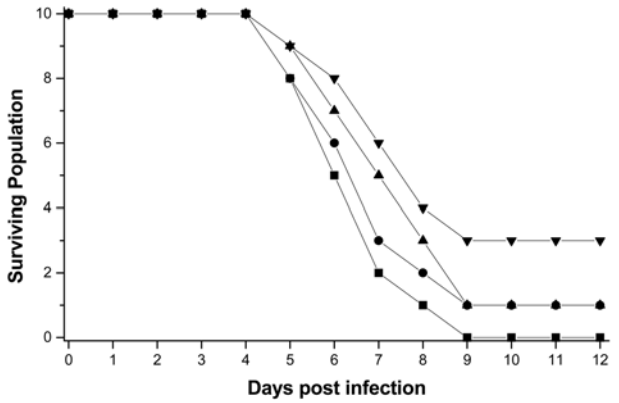

Fig. 2. Mortality rate of mice infected with *S. typhimurium* after treatment of Smectite, CLP1000 and CLPS1000. ■ , control group treated with phosphate buffer solution (PBS); ● , the group treated with 10 mg/ml Smectite (n=10); ▲ , the group treated with 10 mg/ml CLP1000 (n=10); ▼ , the group treated with 10 mg/ml CLPS1000 (n=10).

depending on the incubation time. At the concentration of 1.0 mg/ml, the bacteriocidal effect of CLP1000 and CLPS1000 was observed on *S. typhimurium*. Kwon *et al.*[4] reported that the combination of methanolic extrats from *Coptidis Rhizoma*, *Mume Fructus*, and *Schizandrae Fructus* had an antimicrobial effect on the *Salmonella* strains, of which the minimum inhibitory concentrations against *S. typhimurium* originated from pig varied from 1.9 to 7.8 mg/ml. Compared with the study by Kwon *at al.*[4], antibacterial activity of CLP100 and CLPS100 in this study may be more effective against *S. typhimurium*.

CLP1000 and CLPS1000 treatment for murine salmonellosis

The therapeutic effects of Smectite, CLP1000, and CLPS1000 against *S. typhimurim* were shown in Fig. 2. The mortality of mice infected with *S. typhimurium* was counted during the experimental period. The mortality rate in the untreated CLP1000 and CLPS1000 was 100% at the 9th day. However, the group treated with 10 mg/ml Smectite, CLP1000, and CLPS1000 survived one, one, and three mice until the 12th day, respectively. And the mortality rate in the group treated with Smectite, CLP1000, and CLPS1000 was 90%, 90%, and 70%, respectively. According to the previous research[4], the extract mixture of *Coptidis Rhizoma*, *Mume Fructus*, and *Schizandrae Fructus* (5:3:2) at the dose of 10 mg/g feed significantly reduced the mortality of chicken infected with *Salmonella gallinarum* by 50%. The mortality rate of the extract mixture fed group was 33%, and that of the non-extract mixture fed group was more than 83%. In the present study, the mortality rate of the treated CLPS1000 at the dose of 10 mg/ml was higher than that of the research carried out by Kwon *et al*[4]. It assumed that the different result of mortality rate was depended on experimental conditions.

In conclusion, our results demonstrate that Smectite, CLP1000, and CLPS1000 at the concentration of 10 mg/ml takes effect against *S. typhimurium*, and at the dose of 10 mg/ml, CLPS1000 possesses the therapeutic effect for the infection of *S. typhimurium* in mice.

요 약

본 연구는 황련, 금은화, 그리고 백작약 혼합분말(1:1:1)의 메탄올 추출물(CLP1000)과, 이에 dioctahedral smectite를 혼합한 합제(CLPS1000)의 살모넬라에 대한 항균효과를 평가하기 위해 수행되었다. CLP1000와 CLPS1000는, 0.5 mg/ml 농도에서 *S. typhimurium*에 대한 항균효과를 보이지 않았으나, 1.0 mg/ml의 농도에서는 *S. typhimurium*에 대한 항균활성이 관찰되었다. BALB/c 마우스를 이용한 살모넬라 감염시험에서 Smectite, CLP1000 그리고 CLPS1000을 각각 10 mg/ml 농도로 12일 동안 투여한 결과, 사망률이 각각 90%, 90% 그리고 70%을 보여, CLPS1000이 마우스 살모넬라증에 강력한 효과를 갖고 있는 것으로 나타났다.

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