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<Original Article>

Effect of a mixture of *Galla rhois* and *Cinnamomum cassia* extracts on susceptibility to the colonization of *Campylobacter jejuni* in broiler chickens

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Abstract : The present study evaluated the effects of a mixture of *Galla rhois* and *Cinnamomum cassia* extracts (GCE) (1 : 1, w/w) on susceptibility to the colonization of *Campylobacter* (*C.*) *jejuni* in broilers. Eighty two-week-old broilers (n = 20 per group) were used to estimate the efficacy of GCE against *C. jejuni* infection via drinking water. Antibacterial activity testing revealed that the minimum bactericidal concentration of GCE against *C. jejuni* was 2.5 mg/mL. Broilers challenged with *C. jejuni* were administered 0.0 (Non-GCE), 2.5 (GCE-2.5), 5.0 (GCE-5.0) and 10.0 g/L (GCE-10) GCE for 7 days, and the cecal contents were collected from five broilers per group on the 1st, 3rd, 5th, and 7th day post-treatment. On day 3 post-administration, the number of *C. jejuni* in GCE-5.0 ($p < 0.05$) and GCE-10 ($p < 0.01$) was significantly decreased relative to Non-GCE, while on day 7 those in all GCE-treated groups were significantly decreased compared to the Non-GCE group ($p < 0.001$). Hematological and blood biochemical analysis revealed no significant differences in parameters between the Non-GCE and GCE-treated groups. Based on the results of the present study, GCE was identified as a safe and alternative candidate to suppress *C. jejuni* colonization in broilers.

Keywords : *Campylobacter jejuni*, *Cinnamomum cassia*, *Galla rhois,* broiler chicken

Introduction

Campylobacteriosis is a food- and water-borne zoonotic diarrheal illness caused by the genus *Campylobacter*, with approximately 90% of the cases caused by *Campylobacter* (*C.*) *jejuni* which is a gram-negative pathogen that grows in low-oxygen environments, including the digestive tracts of animals [1, 24]. Endemically, *C. jejuni* colonizes commercial chicken flocks, and human disease is usually linked to the ingestion of food cross-contaminated with raw or undercooked poultry [20]. After ingestion of contaminated food, human campylobacteriosis develops within 2-3 days and symptoms usually resolve within a week, and sequelae, including autoimmune-mediated demyelinating neuropathies such as Guillain-Barre and Miller Fisher syndromes, may occur [23].

In the European Union, the number of human campylobacteriosis cases has followed a significant increasing trend in the last several years, with 220,209 cases confirmed in 2011 [4]. In the United States, approximately 1.3 million symptomatic *Campylobacter* infections occur annually, and the majority of *Campylobacter* infections are acquired via the oral route after handling raw poultry or consuming uncooked poultry [29]. In Korea, the annual incidence of *Campylobacter* infections was estimated as 2,196 cases during 2008–2012 [21].

C. jejuni naturally colonizes a wide range of domestic and wild animals, especially poultry, which are recognized as the main reservoir of the microorganism and rarely show clinical signs of disease [7, 10]. In particular, chickens and turkeys carry high concentrations of *Campylobacter* in their intestinal tract, posing a high risk of carcass contamination during the slaughtering process. In addition, handling and consumption of contaminated poultry meat are the major source of human campylobacteriosis [10].

Following the widespread use of antimicrobial drugs in intensive animal production for growth promotion and prevention or treatment of disease, *Campylobacter* spp. have recently developed resistance to many clinically important antimicrobials, including fluoroquinolones [26]. The transmission and spread of antibiotic-resistant *Campylobacter* spp.

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can not only be affected by environmental and host factors, but can also be influenced by the relative fitness of the drug-resistant bacteria in the absence of selection pressure [18].

After a ban on the use of sub-therapeutic antimicrobials in feed, more therapeutic antimicrobials were needed in many European countries to control animal diseases and keep animals healthy [8]. The increased amount of therapeutic antimicrobials was equal to or even more than the total quantity of antimicrobials being used before the ban on growth-promoting antibiotics [22].

Recently, many researchers have carried out studies on alternative antibiotics for the control of *C. jejuni* using probiotics [6], organic acids [27], bacteriophage [13] and medicinal plants [16].

Galla rhois (GR) has long been applied in traditional Oriental medicine for the treatment of diarrhea, persistent coughing and spontaneous perspiration in humans, as a result of the effects of astringents, antidiarrheals, hemostatic drugs and other antidotes [17]. GR is a natural, non-toxic material that contains a number of tannin-derived components, collectively termed tannic acid, methyl gallate and gallic acid. Notably, the gallotannins are a class of hydrolysable tannin polymers that are formed from gallic acid, which seems to possess anti-bacterial, anti-fungal, and anti-viral properties [16, 17].

Cinnamomum cassia (CC) is a type of deciduous tree that grows in Korea, China, and Japan. In Korea, CC has long been ethnopharmacologically used as a folk medicine to treat various inflammatory diseases and respiratory tract disease [11]. CC has several proven biological activities, including anti-bacterial and anti-viral activity, anti-inflammatory effect, and anti-tumor activity [5, 11]. In the essential oil of CC, cinnamaldehyde which possesses broad-spectrum antibiotic activities, is the major component (comprising 85%) [5].

Although previous studies [3, 5, 15] investigated the antibacterial effects of an extract of CC against *C. jejuni*, few studies have investigated the effect of a combination of GR and CC on broiler chickens. The present study evaluated the control potential of a combination of GR and CC extract against *C. jejuni* in broiler chickens.

Materials and Methods

Preparation of a combined herbal extract

GR and CC were purchased from the Korea National Animal Bio Resource Bank (Jinju, Korea) and were air-dried in a dark room and ground to a powder. One hundred g of each of GR and CC powder was soaked in 400 mL of 70% aqueous methanol (methanol/water = 70/30, v/v) for 24 h under mantle-reflux. The solvent was removed under reduced pressure in a rotary evaporator (N-1000 S; Tokyo Rikakikai, Japan). The extracts were filtered using Whatman No. 1 filter paper, and the filtrates were freeze-dried using a vacuum freeze dryer (MCFD 8508; ilShinBioBase, Korea) and blended into powder using a mill (Kinematica, Switzerland) with 90 standard mesh. The extract powders were mixed with the inverse ratio (1 : 1, w/w) of each extract of minimum bactericidal concentration (MBC) against *C. jejuni*, and the combination was designated as GR and CC extracts (GCE).

Bacterial strains and culture conditions

C. jejuni strain ATCC 33291 was used for all experiments. According to the previous method [9], the bacteria were routinely cultured in Nutrient Broth No. 2 (NB 2; Oxoid, UK) supplemented with Modified Preston Campylobacter Selective Supplement (MPC; Oxoid, UK) and Campylobacter Growth Supplement (CGS; Oxoid), at 42°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank's balanced salt solution (HBSS; Gibco-BRL, USA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid) supplemented with CCDA Selective Supplement (Oxoid) and CGS, followed by microaerobic incubation at 42°C for 22 h.

Antibacterial activity of herbal extracts

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and MBC of GR and CC extracts against *C. jejuni*. All tests were performed in Mueller-Hinton broth (MHB; BD Difco, USA). Briefly, serial doubling dilutions of the herbal extracts, ranging from 0.25 to 5.0 mg/mL, were prepared in a 96-well microtiter plate. To each well, 20 μL of indicator solution (prepared by dissolving a 10-mg extract in 1 mL of phosphate buffer solution) and 80 μL of MHB were added. Finally, 100 μL of bacterial suspension (10^6 colony-forming unit [CFU]/mL) were added to each well to achieve a concentration of 10^5 CFU/mL. The plates were prepared in triplicates, and then placed in an incubator at 42°C for 48 h under microaerobic conditions. After incubation, the lowest concentration at which the microorganism does not demonstrate visible growth was taken as the MIC value. Afterwards, 10 μL of each well were transferred to Mueller-Hinton agar containing 5% sheep blood and incubated at 42°C for 48 h under microaerobic conditions. The lowest concentration associated with no visible growth of bacteria on the agar plates was considered the MBC. All dilutions were performed in triplicate.

Animals

The animal care and use protocol was approved by the Animal Ethical Committee of Gyeongsang National University Institutional Animal Care and Use Committee (approval No. GNU-120614-R0125).

A total of 80 one-day-old Ross chickens obtained from Harim (Korea), were grown over a 3-week experimental period. The chicks were kept in brooders with wire screen floors for 2 weeks. At 2 weeks of age, all chicks (421 ± 35 g, body weight) were individually weighed and tagged and randomly assigned to 4 groups consisting of four replicates (cages), each with 5 individuals. The four experimental groups were as follows: chicks treated with normal water without GCE

(Non-GCE), chicks treated with GCE 2.5 g/L via drinking water (GCE-2.5), chicks treated with GCE 5.0 g/L via drinking water (GCE-5.0) and chicks treated with GCE 10.0 g/L via drinking water (GCE-10). The room was illuminated continuously with ambient temperature maintained at 24 ± 1°C until the end of the experiment. Chicks were allowed *ad libitum* access to both the commercial starter diet (Purina Feed, Korea) and water throughout the 14-day experimental period.

C. jejuni challenge and administration of GCE

The *Campylobacter* challenge test was carried out according to the previous study [6]. All chicks were orally challenged with 1×10^5 CFU of *C. jejuni*. On day 5 post-challenge, GCE-2.5, 5.0 and -10 groups were administered with 2.5, 5.0 and 10.0 g/L of GCE in drinking water, respectively, for 7 consecutive days. In addition, Non-GCE was administered with normal water without GCE.

Enumeration of *C. jejuni* numbers in cecal contents

On days 1, 3, 5 and 7 post-treatment with GCE, five chicks in each group were euthanized by cervical dislocation. Continually, cecal contents were aseptically collected from the chick at autopsy. After homogenization of 0.2 g of each cecal content by adding 1.8 mL of NB 2 supplemented with MPC and CGS, the homogenate was serially diluted 10 times with HBSS from 10^{-2} to 10^{-10}. One hundred μL of each diluent were spread onto mCCDA supplemented with CCDA selective supplement and CGS, and the plates were incubated under microaerobic conditions at 42°C for 48 h. After incubation, representative colonies which were confirmed as *C. jejuni* by both phase-contrast microscopy and latex agglutination tests, were counted and represented as CFU/g.

Blood analysis

To determine the safety of GCE, the hematological analysis was carried out using the blood collected from broiler chickens at the end of the experiment. The birds were starved for 12 h and blood was collected from the wing vein of 5 chicks per treatment group with the aid of needle and syringe. Blood samples (5 mL) were collected from each chicken and transferred immediately into a set of sterile tubes (Becton, Dickinson and Company, USA) with and without anti-coagulant for hematological and serum biochemical tests respectively. The whole blood sample was analyzed for white blood cell count, red blood cell count, hematocrit and hemoglobin using an Advia 120 hematology analyzer (Bayer, USA). For the analysis of serum biochemical values, blood samples were centrifuged at 5,000 × g for 10 min. Alanine aminotransferase and aspartate aminotransferase activities, blood urea nitrogen and creatinine concentrations in the serum were determined using a Hitachi 911 chemistry analyzer (Roche Diagnostics, Korea).

Statistical analysis

All results were expressed as the mean ± SD. The data were analyzed using an one-way analysis of variance (ANOVA) and Student's *t*-test. The p value < 0.05 was considered to be statistically significant.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of herbal extract alone and herbal extract complex against *Campylobacter jejuni*

Extracts	MIC (mg/mL)	MBC (mg/mL)
Galla rhois	0.5	4.0
Cinnamomum cassia	2.0	4.0
GCE	1.5	2.5

GCE, a mixture of *Galla rhois* extract and *Cinnamomum cassia* extract (1 : 1, w/w).

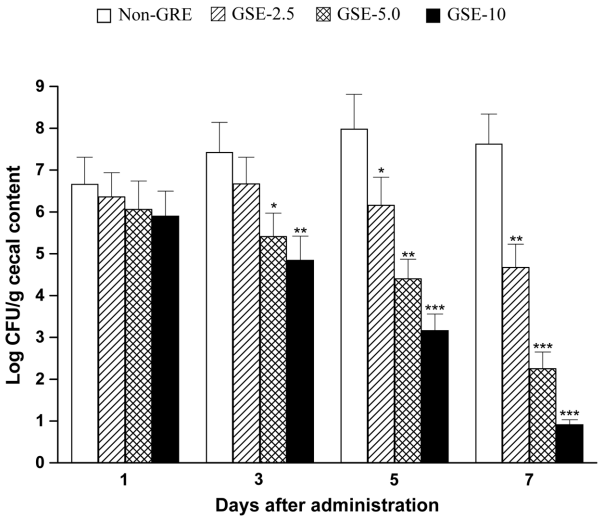

Fig. 1. *Campylobacter jejuni* counts in cecal contents of broiler chickens administered with different concentrations of GCE via drinking water. Significantly different from Non-GCE at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Antibacterial activity of GCE

As shown in Table 1, the MIC of GR extracts against *C. jejuni* was lowest, followed by GCE and CC extract. However, the MBC of GCE against *C. jejuni* was lowest, followed by GR and CC extracts, which was both 4.0 mg/mL. As GCE was blended with the inverse ratio of MBC of GR and CC extracts, the combined extracts contained 50% of GR extracts and 50% of CC extracts.

Change in *C. jejuni* numbers in cecal contents

Figure 1 shows the change in *C. jejuni* numbers in cecal contents of broilers administered with different concentrations of GCE during the experimental period. On day 1 post-treatment, *C. jejuni* numbers in groups treated with GCE were not meaningfully reduced compared with those in Non-

Table 2. Blood cell counts and biochemical values in broilers treated with various concentrations of herbal extract complex

Parameters	Normal range	Experimental groups			
		Non-GCE	GCE-2.5	GCE-5.0	GCE-10.0
RBC (M/mm^3)	5.00–8.00	6.29 ± 0.24	6.43 ± 0.29	6.32 ± 0.34	6.37 ± 0.31
WBC (M/mm^3)	12.00–30.00	16.27 ± 1.53	16.31 ± 1.44	16.26 ± 1.48	16.41 ± 1.50
HCT (%)	22.0–35.0	26.9 ± 2.17	27.1 ± 1.87	26.8 ± 2.23	27.3 ± 2.04
Hb (g/dL)	7.0–13.0	9.2 ± 0.65	9.7 ± 1.15	9.1 ± 0.76	9.5 ± 0.83
AST (IU/L)	150–400	290 ± 17.4	294 ± 15.7	289 ± 18.1	292 ± 21.2
ALT (IU/L)	0–668	36 ± 3.75	37 ± 4.21	36 ± 3.75	37 ± 4.26
BUN (mg/dL)	0–124	32 ± 3.63	30 ± 4.28	28 ± 3.65	31 ± 3.91
Creatinine (mg/dL)	0.1–0.4	0.35 ± 0.08	0.39 ± 0.06	0.3 ± 0.11	0.3 ± 0.07

Non-GCE, no treatment; GCE-2.5, GCE 2.5 g/L in drinking water; GCE-5.0, GCE 5.0 g/L in drinking water; GCE-10, GCE 10.0 g/L in drinking water; RBC, red blood cell; WBC, white blood cell; HCT, hematocrit; Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

GCE. On day 3 post-administration, *C. jejuni* counts in GCE-5.0 ($p < 0.05$) and -10 ($p < 0.001$) were significantly decreased compared with those in Non-GCE. On day 5 after treatment, the number of *C. jejuni* in all groups treated with GCE (GCE-2.5, $p < 0.05$; GCE-5.0, $p < 0.01$; GCE-10, $p < 0.001$) was significantly reduced compared with that in Non-GCE. On day 7 post-administration, the number of *C. jejuni* in all groups treated with GCE was significantly reduced compared with that in Non-GCE ($p < 0.001$).

Blood cell counts and serological values

The analytical results of the hematological and blood biochemical indices on broiler chickens treated with various doses of GCE were presented at Table 2. Hematological and blood biochemical parameters in all GCE-treated groups were within the normal range. In addition, no significant differences in index values between Non-GCE and GCE-treated groups were observed. There was also no dose-response relationship for any parameters between GCE-treated groups.

Discussion

The antibacterial effect of herbal extracts against *C. jejuni* has been well reported in many previous studies [3, 5, 15], but few studies have investigated the antibacterial effect of a combination of herb extracts against *C. jejuni*. The present study was carried out to investigate the anti-*Campylobacter* activity and inhibitory effect on cecal colonization of GCE in broiler chickens challenged with *C. jejuni*. In a previous study on the synergistic effect of antibacterial plant extracts [12], the inhibitory zone (IZ) of a combination of *Leucas aspera* and *Lobelia nicotianaefolia* extract was 2.1 cm against *Staphylococcus aureus* although IZ of *Leucas aspera* and *Lobelia nicotianaefolia* extract was 0.8 and 1.5 cm, respectively. In the present study, the MBC of GCE against *C. jejuni* was lower than that of GR and CC extract, which might be due to the interactive effect of GR or CC extracts, as indicated in the study above.

In another previous study [15], the MIC value of aqueous Chinese leek extract against *C. jejuni* was 2.0 mg/mL. In preceding studies on the anti-*Campylobacter* effects of herbal extracts, the MBC of *Eleutherine americana* Merr. extract against *Campylobacter* spp. ranged from 31.25 to 1,000 μg/mL [25], and that of *Foeniculum vulgare* and *Zingiber officinale* extract against *C. jejuni* was the same at 20 mg/mL [3]. Considering the herbal extract method and bacteria species, the MIC and/or MBC of GCE in the present study were lower than those of the above herbal extracts, except for the *Eleutherine americana* Merr. extract.

Based on the results of the present study, it appears that GCE, which contains GR and CC extracts, was successful in reducing the cecal colonization of *C. jejuni* in broiler chickens. According to the previous study [19], the broiler group administered a mixture of powdered stems, leaves, capsules and seeds of *Macleaya cordata* with 0.5 g/kg feed for 28 days, showed significant reduction of *C. jejuni* counts in cecal contents compared to the control group ($p < 0.05$). In addition, another previous study [27] reported that broiler groups treated with caprylic acid in their feed at concentrations of 0.7 or 1.4% for 3 days, showed a significant decrease of *C. jejuni* counts in the cecum compared with the control group ($p < 0.05$). However, no reduction in *C. jejuni* counts was observed in the cecal contents of broiler chickens administered with a bacteriophage cocktail ($\log_{10}$ 7.5 PFU/bird for 7 days) [13] or *Acacia decurrens* extract (0.5-1.0 g/kg feed, for 45 days) [14]. Considering the extract solvents, and route of administration and treatment period, the efficacy of GCE in reducing *C. jejuni* cecal colonization was slightly higher than or similar to those of a mixture of different parts of *Macleaya cordata*, a bacteriophage cocktail and *Acacia decurrens* extract, but was lower than that of caprylic acid because of the high dose as a synthetic chemical.

To identify the toxic effect of GCE, hematological and blood biochemical values were analyzed using the collected blood at the end of the experiment. No parameter values were significantly different between Non-GCE and GCE-

treated groups. In a previous study [2], all hematological and serum biochemical parameters in the blood of piglets treated with GR extract (4.0 g/kg feed for 28 days), were within the normal physiological range. According to another previous study [28], no significant differences were observed in any of the hematological and serum biochemical parameters of broiler chickens administered with cinnamon (4.0 g/kg feed for 42 days), compared with those of the control. In this GCE-10 group, the concentration of GR and CC extracts was both 5.0 g/L and the period of treatment was 7 days. Therefore, the amount of GR and CC extract intake was absolutely lower than that of above studies, although the level of GR and CC extracts was slightly higher than that of the aforementioned studies. Thus, the results of hematological and blood biochemical analyses in the present study were in agreement with those in the preceding studies.

In conclusion, the results in the present study demonstrate that GCE at a concentration of 2.5 g/L in drinking water may be used to suppress *C. jejuni* colonization in broiler chickens. Therefore, GCE could be a safe and effective candidate for the treatment of *C. jejuni* infection in broiler chickens.

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