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In Vitro Activity of Methyl Gallate Isolated from Galla Rhois Alone and in Combination with Ciprofloxacin Against Clinical Isolates of *Salmonella*

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***Salmonella* remains a primary cause of food poisoning worldwide, and massive outbreaks have been witnessed in recent years. Therefore, this study investigated the antimicrobial activity of methyl gallate (MG), which exhibited good antibacterial activity (MIC=3.9–125 μg/ml) against all the bacterial strains tested. In a checkerboard dilution test, MG markedly lowered the MICs of ciprofloxacin (CPFX) against *Salmonella*. The combined activity of CPFX and MG against *Salmonella* resulted in fractional inhibitory concentrations (FICs) ranging from 0.0037 to 0.015 and from 0.24 to 7.8 μg/ml, respectively. Meanwhile, the FIC index ranged from 0.31–0.37, indicating a marked synergistic relationship between CPFX and MG against *Salmonella*. Time-kill assays also showed a decrease in the CFU/ml between the combination and the more active compound. Therefore, this study demonstrated that MG and CPFX can act synergistically in inhibiting *Salmonella in vitro*.**

Keywords: *Salmonella*, ciprofloxacin, methyl gallate, synergism

Salmonella enterica is the primary bacterial agent causing foodborne infections in humans all over the world [14]. Certain pathogenic *Salmonella* serotypes that have adapted to humans, including *S. typhimurium*, invariably cause severe diseases such as enteric fever. Serotypes that are highly adapted to animal hosts, such *S. gallinarum* (poultry), rarely cause disease in humans. *S. dublin* has a preference for cattle and is primarily responsible for fever, a reduced milk yield, diarrhea, abortion, and high mortality [9]. A recent enteric fever epidemic was reported to result from infection by *S. enterica* serovar typhi with resistance to ciprofloxacin (CPFX), a fluoroquinolone introduced for the treatment of enteric fever over a decade ago [1].

Ciprofloxacin is the drug of choice for treating typhoid fever in areas where multidrug-resistant (MDR) *Salmonella* strains are prevalent [17]. Thus, such reports of infections due to strains of *Salmonella* with a high-level resistance to fluoroquinolones are particularly worrying. The emergence of complete resistance to ciprofloxacin in *Salmonella* would severely limit the choice of antimicrobial therapies for treating enteric fever. Consequently, there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy [13].

Galla Rhois has long been used in traditional Korean medicine and other Oriental medicine systems for the treatment of diarrhea, prolonged coughing, and spontaneous perspiration. Galla Rhois is a natural non-toxic and contains several tannin-derived components, such as methyl gallate (MG) and gallic acid. MG is also known to possess growth-inhibiting activity against *E. coli* without adversely affecting the growth of lactic acid-producing bacteria [2, 3].

Accordingly, the present study was performed to assess the effects of combining CPFX and MG against *Salmonella* isolates that are both unresponsive to antibiotics and show high minimum inhibitory concentrations (MICs) *in vitro*.

Galla Rhois, purchased from the Oriental drug store Daehak Hanyak kuk (Iksan, Korea), was authenticated by Dr. D. Y. Kwon. A voucher specimen (No. 06-021) was also deposited in the Laboratory of Herbalogy, College of Pharmacy, Wonkwang University, Iksan, Korea. The EtOH extracts were partitioned using organic solvents with different polarities to yield *n*-hexane, EtOAc, *n*-BuOH, and H_2O fractions in sequence. The EtOAc fraction from each plant was then subjected to silica gel chromatography with $CHCl_3$-MeOH-H_2O (lower layers, by volume, 25:7:5, 7:3:1)

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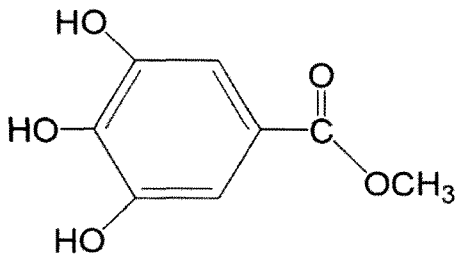

Fig. 1. Structure of methyl gallate isolated from Galla Rhois.

as the solvent to yield MG (Fig. 1) from Galla Rhois. The structure of the compound was determined by its physicochemical and spectral data (^{1}H-NMR and ^{13}C-NMR), which agreed with those previously reported [2].

S. gallinarum (ATCC 9184) was also used in this study (Table 1), along with local isolates of *S. enteritidis*, *S. gallinarum*, and *S. typhimurium* provided by the National Veterinary Research and Quarantine Service, Republic of Korea. The bacterial strains were suspended in a Mueller-Hinton broth (MHB; Difco, U.S.A.) and incubated at 37°C for 20 h. A Mueller-Hinton agar (MHA; Difco, U.S.A.) was then used for the MIC. The resistance of the *Salmonella* strains to the two antimicrobial agents was determined using the disk-agar method standardized by the Clinical and Laboratory Standards Institute (CLSI) [11]. The quality control strains were *Enterococcus faecalis* ATCC 29212 and *E. coli* ATCC 25922 (Table 1). The MIC values were determined for the microorganisms found to be sensitive to MG during the disk diffusion assay. Microorganism inocula were prepared from 12-h broth cultures and the suspensions adjusted to a 0.5 McFarland standard turbidity. Susceptibility tests were carried out using the standard broth microdilution method in accordance with CLSI guidelines [11] in MHB with an inoculum of approximately 5×10^4 CFU/ml. The MHB was supplemented with serial CPFX concentrations ranging from 0.0005 to 3.9 μg/ml, and MG at concentrations from 0.97 to 1,000 μg/ml. The data were reported as the MICs, representing as the lowest concentration of CPFX and MG inhibiting visible growth after 24 h of incubation at 37°C [10, 19]. The MICs for CPFX were also determined, and similarly defined as the lowest antibiotic concentration at which no visible bacterial growth was observed.

The antibacterial effects that resulted from combining the two antimicrobial agents were assessed using the checkerboard test. The antimicrobial combination assayed included MG plus CPFX. Serial dilutions of the two antimicrobial agents were mixed in cation-supplemented MHB. The inocula were prepared from colonies that had been grown on MHA overnight. The final bacterial concentration after inoculation was 5×10^4 CFU/ml. The MIC was determined after 24 h of incubation at 37°C. Each experiment was repeated three times. The fractional inhibitory concentration (FIC) index was determined using the following formula: FIC index=$FIC_A+FIC_B=[A]/MIC_A+[B]/MIC_B$, where [A] is the concentration of drug A, MIC_A is its MIC, and FIC_A is the FIC of drug A for the organism, and [B], MIC_B, and FIC_B are defined in the same way for drug B. The FIC index thus obtained was interpreted as follows: <0.5, synergy; 0.5 to 0.75, partial synergy; 0.76 to 1.0, additive effect; >1.0 to 4.0, indifference; and >4.0, antagonism [21]. Finally, the varying rates of synergy between the two agents were determined by performing a Chi-square analysis [16]. A time-kill curve assay was also performed according to the method described by Chang and others [5] to study the combined effects of time and the antimicrobial agent concentration on bacterial growth. Here, cation-supplemented MHB was used that included either a single antimicrobial agent or a combination of the two drugs. The concentration of each antimicrobial agent in the media was set at a level equal to

Table 1. *Salmonella* strains used in this study and growth inhibition zones produced by antibiotics.

Strains	Serotypes	Origin	[a]Resistant antibiotics	Diameter of clear zone (mm)								
				AM 10 μg	AMC 20 μg	C 30 μg	CF 30 μg	NA 30 μg	NOR 10 μg	S 10 μg	SXT 2 μg	TIC 75 μg
WPH 1	*S. gallinarum* ATCC 9184	Chicken	AM, AMC, C, S, TIC	<8	10	12	27	>30	>30	8	28	13
WPH 2	*S. gallinarum*	Chicken	CF, NA, NOR, S, SXT	27	>30	>30	8	12	11	8	10	>30
WPH 3	*S. gallinarum*	Chicken	NA, S	>30	>30	28	>30	8	>30	12	28	>30
WPH 4	*S. typhimurium*	Cattle	-	28	>30	28	>30	>30	>30	>30	>30	>30
WPH 5	*S. typhimurium*	Pig	AM, AMC, S, TIC	<8	8	>30	>30	>30	>30	<8	27	11
WPH 6	*S. enteritidis*	Chicken	-	27	>30	28	>30	>30	>30	>30	>30	>30
WPH 7	*S. enteritidis*	Chicken	-	28	>30	>30	>30	>30	>30	>30	>30	>30

AM, ampicillin; AMC, amoxicillin/clavulanic acid; C, chloramphenicol; CF, cephalothin; NA, nalidixic acid; NOR, norfloxacin; S, streptomycin; SXT, trimethoprim/sulfamethoxazole; TIC, ticarcillin.

[a]The antibiotic resistance was reflected in the diameter of the clear zone (mm) values for AM (≤13 mm), AMC (≤13 mm), C (≤12 mm), CF (≤13 mm), NA (≤13 mm), NOR (≤13 mm), S (≤10 mm), SXT (≤10 mm), and TIC (≤14 mm) [11].

Table 2. Result of combined effect of methyl gallate (MG) and ciprofloxacin (CPFX) against *Salmonella*.

Strains	Serotype	Origin	[a]MIC MG (μg/ml)		MIC CPFX (μg/ml)		[b]FICI	Outcome
			Alone	With CPFX	Alone	With MG		
WPH 1	*S. gallinarum* (ATCC 9184)	Chicken	31.25	7.8	0.06	0.0075	0.37	Synergistic
WPH 2	*S. gallinarum*	Chicken	3.9	0.24	0.06	0.015	0.31	Synergistic
WPH 3	*S. gallinarum*	Chicken	15.6	1.97	0.06	0.015	0.37	Synergistic
WPH 4	*S. typhimurium*	Cattle	3.9	0.24	0.015	0.0037	0.31	Synergistic
WPH 5	*S. typhimurium*	Pig	125	7.8	0.03	0.0075	0.31	Synergistic
WPH 6	*S. enteritidis*	Chicken	125	7.8	0.03	0.0075	0.31	Synergistic
WPH 7	*S. enteritidis*	Chicken	31.25	7.8	0.03	0.0037	0.37	Synergistic

[a]MIC, Minimum inhibitory concentration.
[b]FICI, fractional inhibitory concentration index.

the MIC of the tested strain. The bacterial inocula consisted of 10^5 CFU/ml and were harvested from colonies grown overnight. The inoculated tubes were incubated at 37°C and continuously agitated. A small amount of the medium was initially removed to count the viable bacteria prior to shaking, and then at intervals of 4, 8, and 24 h after starting the incubation process. According to the method of Chang *et al.* [5], if the final concentration of viable bacteria growing on the media containing the two antimicrobial agents was 100-fold less than the concentration of bacteria growing on the media containing a single antimicrobial agent, synergy was considered to be present. Conversely, if the final concentration of viable bacteria on the media containing the two antimicrobial agents was >100-fold higher than the concentration of bacteria growing on the media containing a single antimicrobial agent, the result was regarded as antagonistic. Additive or indifference to the treatment was defined as any other scenario not meeting the criteria for synergy or antagonism. The time-kill experiments were all performed at least three times to confirm the results, and the data presented are the mean±S.D.

The MICs for MG and CPFX against the 7 strains of *Salmonella* are shown in Table 2. The MICs determined using the broth dilution method confirmed the antimicrobial effects. MG was found to exhibit antimicrobial activity against all the tested strains. The MICs for MG against the 7 *Salmonella* strains ranged from 3.9 to 125 μg/ml, whereas the MICs for CPFX ranged from 0.015 to 0.06 μg/ml. The FICIs for MG combined with CPFX ranged from 0.31–0.37 for the 7 strains of *Salmonella* (Table 2). The FICIs for all the tested strains were <0.5. Thus, synergy was noted in 100% of the cases. The time-kill kinetics were evaluated for 3 *Salmonella* strains in which a synergistic activity was observed, and the same synergistic bactericidal profile was exhibited for these strains. Figs. 2, 3, and 4 show the synergistic activity of MG with CPFX against *S. gallinarum* ATCC 9184 (WPH 1), *S. typhimurium* (WPH 5), and *S. enteritidi* (WPH 7), respectively. The time-kill curve study also demonstrated synergistic antimicrobial activity.

Galla Rhois is native to Korea and China, where in the latter case it is known as Chinese Sumac, and has been used

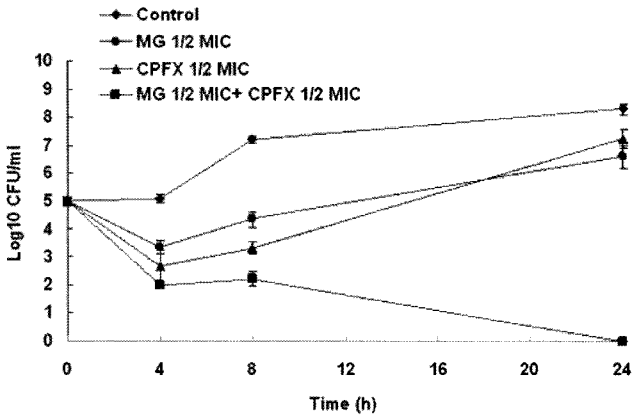

Fig. 2. Time-kill curves for clinical isolate of *S. gallinarum* ATCC 9184 (WPH 1) when using methyl gallate (MG) and ciprofloxacin (CPFX).
●, MG 1/2 MIC (15.6 μg/ml); ▲, CPFX 1/2 MIC (0.03 μg/ml); ■, MG 1/2 MIC (15.6 μg/ml)+CPFX 1/2 MIC (0.03 μg/ml).

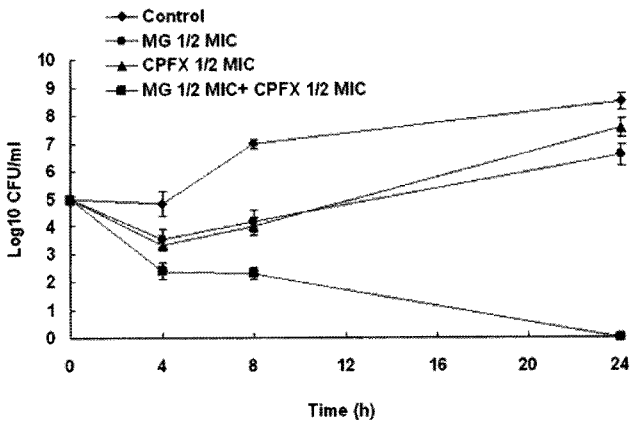

Fig. 3. Time-kill curves for clinical isolate of *S. typhimurium* (WPH 5) when using methyl gallate (MG) and ciprofloxacin (CPFX).
●, MG 1/2 MIC (62.5 μg/ml); ▲, CPFX 1/2 MIC (0.015 μg/ml); ■, MG 1/2 MIC (62.5 μg/ml)+CPFX 1/2 MIC (0.015 μg/ml).

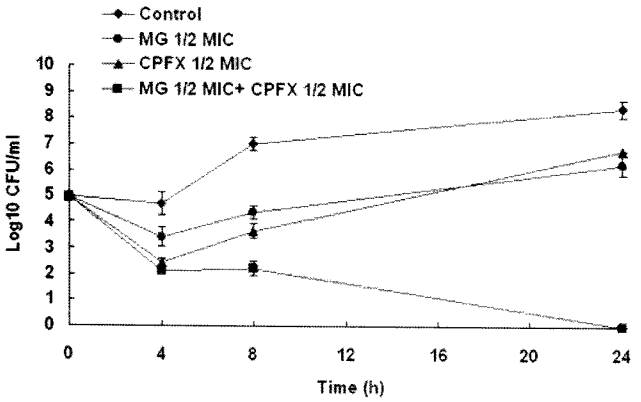

Fig. 4. Time-kill curves for clinical isolate of *S. enteritidis* (WPH 7) when using methyl gallate (MG) and ciprofloxacin (CPFX). ●, MG 1/2 MIC (15.6 μg/ml); ▲, CPFX 1/2 MIC (0.015 μg/ml); ■, MG 1/2 MIC (15.6 μg/ml)+CPFX 1/2 MIC (0.015 μg/ml).

in the treatment of colds, fever, coughs, and malaria. Moreover, the aqueous extract from the gall of *Rhus chinensis* has exhibited activity on alpha-glucosidase [20]. It has been previously reported that the methanol extract of Galla Rhois shows significant growth-inhibitory activity towards both *Clostridium perfringens* and *Escherichia coli*, yet not towards *Bifidobacterium adolescentis* or *Lactobacillus acidophilus* [2]. In East Asia, Galla Rhois has long been considered to have natural medicinal properties, plus it is rich in tannins. MG is a phytochemical from various species, including Meliaceae, Rosa Rugosa, and Galla Rhois, and its derivative (−)-epigallocatechin gallate (EGCG) is a major phytochemical of green tea, a well-known antioxidative beverage [7]. In a continuing effort to identify natural products from Korean plants that inhibit the growth of *Salmonella* or reverse its resistance to current antibiotics, this study evaluated MG's potential to enhance the activity of antibiotics against *Salmonella*. The bacteriostatic and bactericidal concentrations of MG against *Salmonella* were also determined. MG is the main component of Galla Rhois, displaying several biological activities, and it clearly exhibited activity against *Salmonella*. It is also noteworthy that the antibacterial activity of MG described in this study agreed well with results previously reported by others, indicating that the activity of Galla Rhois and other species against *Salmonella* is due to this compound [2, 7, 8, 15].

To overcome the emerging problem of bacterial antibiotic resistance, various studies investigating combinations of plant extracts with antibiotics against clinical strains have been reported [12, 18, 22]. In the present study, combining sub-MIC concentrations of MG with CPFX significantly improved the activity of the antibiotic. The synergistic activity of the CPFX and MG combination was confirmed by time-kill assays, where synergistic activity was observed in the *Salmonella* strains when CPFX was coupled with MG. Thus, MG was shown to have an effect on the CPFX activity, plus the *in vitro* activity of MG against *Salmonella* and its synergistic interactions with CPFX were demonstrated for the first time. Therefore, MG has the potential to restore the effectiveness of CPFX against resistant *Salmonella*, and could be useful in developing valuable clinical treatments.

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